SIGNAL MECHANISMS OF PHOTOTRANSDUCTION IN RETINAL ROD

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I. INTRODUCTION

The rod photoreceptors in the vertebrate retina respond with enormous sensitivity and reliability to light. In total darkness, rods can distinctly signal the arrival of a single photon.[1,2] Equally impressive, however, is that in the presence of continuous light rods can adjust their sensitivity, a process known as light adaptation, and as a consequence the total range of their response extends between 1 and about 10^7 photons.[3] The rods absorb photons with high efficiency in part because their light-gathering photopigment, rhodopsin, exists densely packed in an orderly array of intracellular membranes known as disc membranes. The chromophore in rhodopsin, an 11-cis isomer of retinaldehyde, is isomerized by the absorption of a photon and a sequence of photochemical changes in the photopigment are, thus, initiated (reviewed in References 4 and 5). These photochemical changes in the intracellular disc membranes are, in turn, linked to a change in the electrical potential across the plasma membrane of the rod. The characteristics of the photochemical changes in the visual pigments are now relatively well understood (reviewed in References 4 and 5) as are the electrical events in the plasma membrane of the rod. The chemical and physical events that link one to the other, however, remain much less understood. It is the intention of this review to recapitulate and evaluate some of the recent information that has provided insights into the mechanisms that link visual pigment excitation to the rod's electrical response to light.

This chapter begins with a brief overview of the anatomical features of the rod cell and recent developments in this area and is followed by a discussion of the characteristics of the electrical response of the cells to light. These electrophysiological characteristics are then used as a framework to develop criteria that should be met in identifying internal messengers that may link rhodopsin excitation to the electrical response to light. The roles of calcium ions and cyclic 3′-5′ guanosyl monophosphate (cGMP) as possible internal messengers are then discussed within the framework generated from the electrophysiological data.

II. ANATOMY OF THE ROD CELL

The rods of all vertebrate species are approximately cylindrical in shape and are structurally and functionally polarized such that photon absorption occurs at one end of the cell, the outer segment, while neurotransmitter release occurs at the opposite end, the synaptic terminal (reviewed in Reference 6). Figure 1 is a photomicrograph of an intact rod photoreceptor isolated from the tiger salamander retina. The cell nucleus, mitochondria, Golgi apparatus, rough endoplasmic reticulum, and the general metabolic machinery of the cell are located in the middle of the cell, the inner segment. The outer and inner segments are connected by a small nonmotile cilium, that contains basal bodies and microtubules arranged in a

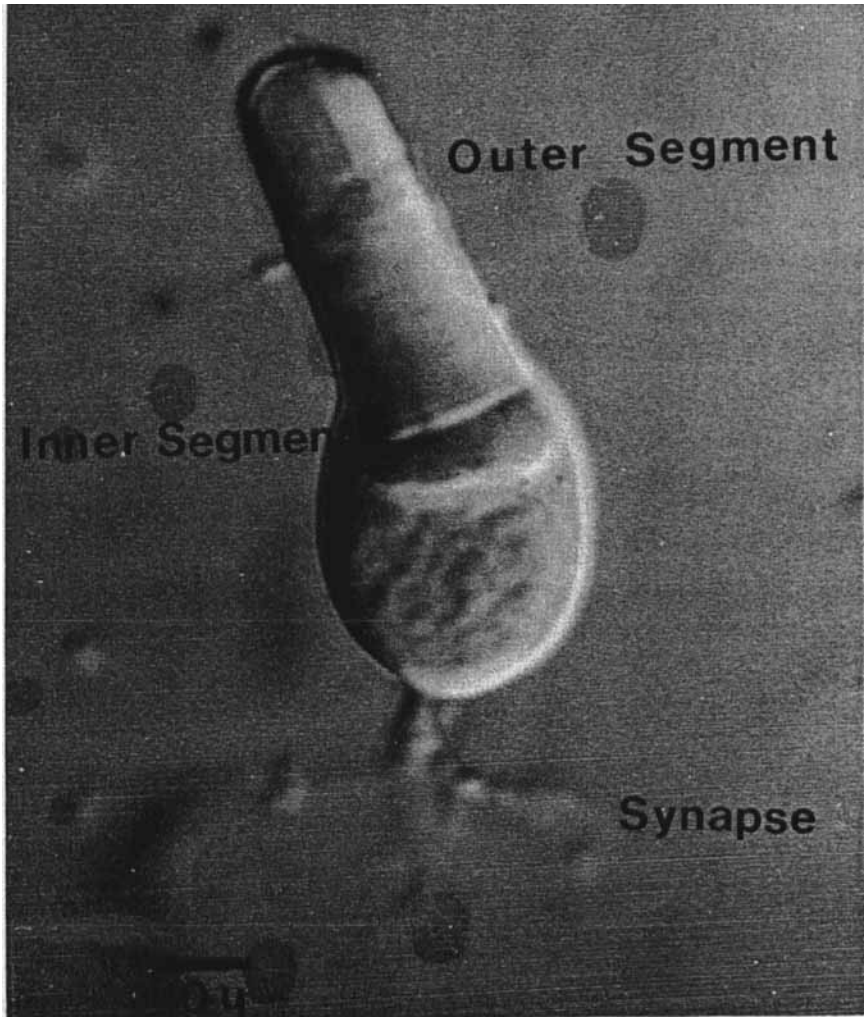

FIGURE 1. Intact rod photoreceptor isolated by enzymatic dissociation of the retina in the tiger salamander (*Ambyostoma tigrinum*). Note the polar organization of the cell with the outer segment at one end and the synaptic terminals at the other. (Photograph courtesy of S. Hestrin.)

typical nine pair circular array. The plasma membrane surrounding the cilium is folded to produce an intricate pattern of folds and ridges known as the paracilliary complex.[7] Despite their common features, the dimensions of rod cells can vary enormously among species. For instance the outer segments are 20 μm long in humans,[6] 200 μm long in some deep-sea fishes,[7] and are 1 μm wide in humans and 15 μm wide in the tiger salamander.

The outer segment consists of a stack of discs, flattened sacs, arranged orderly one above the other and surrounded by a plasma membrane.[6,9] The discs are stacked perpendicular to the long axis of the outer segment and with almost crystalline precision. Discs are approximately 150 Å thick and are spaced with about 300 Å between disc centers.[10-12] Remarkably, disc thickness and spacing are essentially the same in all species investigated to date, independent of disc diameter.[12,13] The discs are not shaped as simple circles; rather they exhibit deep incisures or clefts that run from the perimeter to nearly the center of the disc.[6,14] The incisures vary in number depending on the diameter of the disc. Small diameter discs, like those in humans or rodents have a single incisure while large diameter discs, such as

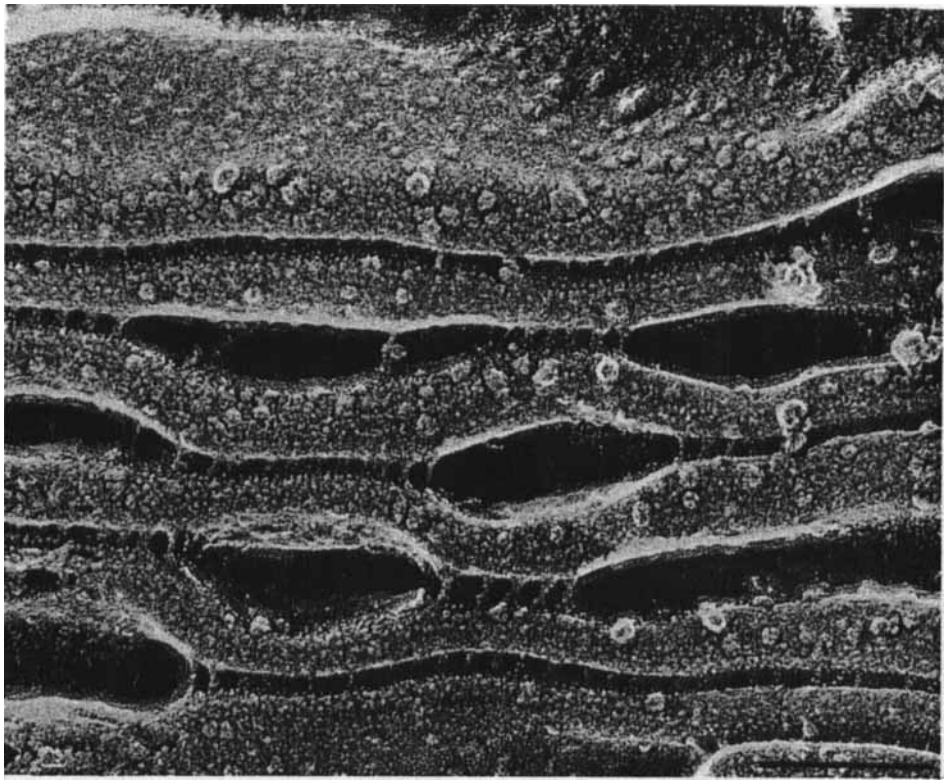

FIGURE 2. Electron micrograph of a metal replica of the freeze-etched cytoplasmic surface of discs isolated from the rod photoreceptor of the toad retina. The large "bumps" on the surface are the G-binding protein. Note that the fibrils that connect the discs to each other are arranged in order around the perimeter of each disc. The fibrils attachment point is recessed approximately 150 Å from the edge of the disc. When discs are separated from each other, the fibrils do not stretch, rather they break. (From Roof, D. I., Korenbrot, J. I., and Heuser, J. E., *J. Cell Biol.*, 95, 501, 1982. With permission.)

those in frogs and toads may have as many as 15.[14] The incisures in the discs are aligned with each other along the length of the outer segment for stretches of several micra at a time. At the end of a given stretch, there occurs a small rotational shift and incisures are in register again at the new angular position until the occurrence of another shift. Microtubule assemblies that originate at the cilium connecting inner and outer segments run in the channels defined by the incisures.[15] The existence of the incisures and their alignment may serve important functions in the cell, particularly where intracellular diffusion of molecules or photoproducts may be critical.

Discs are renewed continuously; new discs are formed at the base of the outer segment, migrate towards the tip, and are finally shed as small packages (reviewed in Reference 16). In frogs the whole outer segment is thereby renewed approximately every 8 weeks. Disc shedding is circadian in higher vertebrates, such as rats, and light entrained in lower vertebrates, such as frogs (reviewed in Reference 17).

The rims of each disc contain structural elements not found elsewhere on the disc which include: (1) a high-molecular-weight protein, approximately 240,000 mol wt embedded in the disc membrane[14] and (2) thin filaments spaced regularly about every 140 Å and set in from the disc edge by about 150 Å (Figure 2).[18] The filaments connect the rim of each disc to the rim of its neighboring discs[18-20] and to the plasma membrane (Figure 3).[18] In addition, the filaments connect the rims of the same disc across the deep incisures. The chemical

FIGURE 3. Electron micrograph of a metal replica of freeze-etched rod outer segments isolated from the retina of the toad. The micrograph illustrates the fibrils that connect the edge of the discs to the plasma membrane. (Photograph courtesy of D. Roof.)

composition of these filaments remains to be determined. Their existence, however, provides a possible structural mechanism for both the observed alignment of disc incisures in the intact outer segment and the reproducible spacing between discs. If free, individual discs would be expected to rotate around their central axis under random thermal diffusion, at a rate of about 20 degrees per hour.[18] Such rotational diffusion is most probably opposed by the anchoring provided by the thin filaments.

The disc membranes are organized as lipid bilayers[10,11] containing proteins both as integral and as peripheral components. Recently, however, freeze fracture studies have shown that within the bilayer order of the disc membranes, small paracrystalline bodies, perhaps indicative of hexagonal lipid phases can be found.[21] The paracrystalline bodies are very rare and their functional significance is unclear. The membrane surfaces of the discs in intact toad rods have recently been visualized under the electron microscope.[18,22] The surface that faces the intradisc space appears to have a subtle structure consisting of 60-Å diameter bumps uniformly distributed at a density of about 30,000/μm^2 and projecting only slightly beyond the plane of the membrane. These are presumed to be projections of the rhodopsin molecules above the plane of the membrane.[18] The cytoplasmic surface of the disc, in contrast, is covered with large particles that project well above the plane of the membrane, range in size from 80 to 120 Å, and are randomly distributed throughout the surface of the membrane at a density of about 2000/μm^2. These particles have been identified as the GTP binding protein involved in the regulation of phosphodiesterase[22] (see below).

III. ELECTROPHYSIOLOGY OF THE ROD CELLS

Just as the rods are structurally polarized, they are also functionally polarized. In the outer segment, illumination modulates an ionic current that flows across the plasma membrane. This current flows through channels that are directly controlled by the transducing processes linked to rhodopsin photoexcitation. The electrical signal generated by changes in current in the outer segment spreads electrotonically to the inner segment where it is modified in time course through the action of membrane ionic currents that are time and voltage dependent, but are not directly controlled by light. In this section, we review some of the functional features of the electrical signal of the photoreceptor, with particular emphasis on the features of the ionic channels of the outer segment.

The electrophysiological characteristics of the plasma membrane of rods are qualitatively similar in all species investigated to date. Because of their relatively large size, the rods in lower vertebrates such as toads (particularly Bufo), frogs (Rana), tiger salamander (Amblyostoma), and turtle (Chelydra or Pseudemyces) have been most extensively investigated, but electrophysiological data have also been obtained in higher vertebrates such as rats and Macaca monkeys. In the following discussion, features common to all cells are emphasized, and specific quantitative details for some species are detailed when appropriate.

A. Electrical Properties of the Plasma Membrane in the Dark

In the dark the rod plasma membrane is principally permeable to K and Na ions.[23-26] The Na permeability alone accounts for about half the total ionic permeability.[25] In addition to Na and K, the cell exhibits proportionately smaller permeabilities to Ca and Cl ions.[24,27-29] Because of these ionic permeabilities, the rod cells maintain a dark membrane potential of 35 to 45 mV negative inside.[23,30-33] This potential is uniform throughout the cell and has the same value in the inner and outer segments.[34] In rods the equilibrium potential for Na ions is about $+5$ to $+10$ mV and for K ions it is about -60 to -65 mV.[35] Thus, at the dark membrane potential there exists a net electromotive force on each of these ions and, as a consequence, a large and sustained ionic current flows across the rod's plasma membrane.[36-38] This "dark current" is a flux of positive charge that originates in the inner segment, flows along the rod, and reenters the cell across the outer segment membrane.[36,37] The dark current is maintained by the sustained activity of ATP dependent and ouabain inhibitable Na/K exchange pumps[36,39] located in the inner, but not the outer segment.[40] The magnitude of the dark current varies, it is about 12 pA in monkeys,[41] 20 pA in toads,[38] 55 pA in tiger salamanders,[42] and 70 pA in rats.[37] There appears to be no simple relationship between outer segment volume (or surface area) and magnitude of the dark current, suggesting that the surface density of membrane sites across which the ionic flux occurs is not the same for all species.

The spatial distribution of the dark current, that is, the fact that it originates at the inner segment and returns into the cell at the outer segment, is a consequence of the nonuniform distribution of ionic permeabilities throughout the cell surface of the rod. The outer segments are highly permeable to Na.[43,116] The Na permeability of a frog rod outer segment membrane is about 2.8×10^{-6} cm/sec,[45] a magnitude comparable to that found in ion-permeable membranes in other cells. In contrast, the inner segment contains several classes of channels with selective permeabilities for K or Cl or Ca, but not Na.[27] The difference in ionic permeabilities of inner and outer segments and the activity of the Na/K pumps present only in the inner segment underly the mechanism of generation of the dark current.[39,43,46,104] The high Na permeability of the plasma membrane permits Na ions to flow into the outer segment down their own electrochemical gradient. The Na ions are then actively extruded from the cell along the inner segment by the Na/K pumps in exchange for the inward transport of K ions. The K ions can then passively flow out of the rod under their electrochemical gradient

across the K-permeable inner segment membrane. Thus, a net positive flow of current is established along the rod length carried principally by K ions along the inner segment and Na ions along the outer segment.

Na ions cross the outer segment plasma membrane through channels* that are controlled by light. K ions cross the inner segment membrane through one of two different K channels which are not light sensitive: one is controlled by voltage and the other is controlled by cytoplasmic free Ca concentration.[27] In tiger salamander rods, the combined conductance of these two K channels in the dark can maintain an outward K current sufficient to balance the Na inward dark current of 55 pA.[27]

In addition to electrical studies, the ionic fluxes across the plasma membrane of the rod in the dark have been studied by osmotic and radiotracer methods. The Na influx into the outer segment in the dark in frogs and toads is about 10^9 Na ions per rod·sec,[43,48] a magnitude that matches well the electrically measured dark current.[38,39] A dark efflux of K ions has also been measured in intact photoreceptors.[49] The measured K efflux must represent the sum of fluxes through the two types of K-permeable channels in the inner segment.

B. Electrical Properties of the Plasma Membrane in the Light

Illumination of the rods results in a decrease of the dark current[36-39,42] and an hyperpolarization of the membrane potential.[23,30-33,50] It is often experimentally convenient to express the suppression of dark current as the appearance of a "photocurrent" opposite in polarity, but identical in amplitude and time course to the suppressed current. The decrease in dark current arises specifically from a decrease in the ion permeability of the outer segment.[23,25,43,47,48,51] That is, light closes the ion channels of the plasma membrane of the outer segment.

1. Characteristics of the Photocurrent

Following a dim and brief flash (5 to 20 msec in duration), the dark current is reduced gradually, reaches a minimum, and then increases again. The time to reach peak varies with the diameter of the outer segment and with temperature: it is fast in the small rods of mammalians at 37°C, approximately 250 msec in rat[52] and monkey,[41] and slower in the large rods of lower vertebrates at room temperature, about 1 to 2 sec in toad and frog rods.[38,39,53] Interestingly, the speed and amplitude of the photocurrent is not uniform along the length of a single rod; in toad rods, for example, the time course and peak amplitude of the photocurrent is faster and larger at the base of the outer segment than at its tip.[38,54]

In complete darkness, the peak amplitude of the peak photocurrent increases linearly with the number of absorbed photons up to the first ten or so photons. It is remarkable to note that in all species tested, the time course of the photocurrent in this linear range (rats,[52] toad red rods,[38] toad green rods,[53] and monkeys[41] can be well fit by adjusting two variable parameters in the following mathematical expression:

$$I(t) = A\,E\left(\frac{t}{\tau}\right)^{n-1}\exp\left(-\frac{t}{\tau}\right) \ldots\ldots \quad (1)$$

where I is the current, A is a proportionality constant, E is the energy of the stimulus flash and n and tau are adjustable parameters. This expression describes the kinetics of the impulse response of a chain of "n" identical exponential processes of time constant tau. Each species has a characteristic value of n and tau. Interestingly, for all species tested "n" has been found to be in the range of 4 to 6 only. A slightly better fit can sometimes be obtained by allowing the exponential processes to have varying rates, within the constraint that rates

* The term "channel" is used to denote the molecular path through which ions cross the biological membranes. There is no implication in the term as to the molecular mechanism of this path; it could either be a pore or a carrier.

increase with the stage number in the ratio a,2a,3a, . . . ,na.[38] It is also notable that the same mathematical formalism describes the time course of the photoresponse of cones[55] and of invertebrate photoreceptors[56] where, indeed, the expression was first described.

The specific molecular mechanisms that give rise to the mathematical formalism described above are unknown. Several possible physical mechanisms could account for the mathematical expression that describes the photocurrent kinetics. Among these, the following deserve particular consideration: (1) the kinetics are controlled by a sequence of "n" first order chemical reactions, each of rate 1/tau; (2) the kinetics are controlled by a molecule that undergoes an "n" sequence of delays each of time constant tau, as might be impossed, for instance, by diffusional delays; (3) the kinetics are controlled by an "n" sequence of intramolecular rearrangements each of time constant tau, as might occur, for instance, in conformational changes in a channel; (4) the kinetics are controlled by the sequential binding of a ligand to "n" sites in a binding molecule, with time constant tau for each binding reaction. Common to all these possible models is that for any sequential step, the forward rate of reaction must be much faster than the backward rate. At this time, none of these or other possible physical models can be excluded. It is unlikely, however, that diffusional delays alone (possibility b) are sufficient to explain the kinetics.[55] Clearly, better understanding of the molecular details of phototransduction is needed to provide a precise physical reality to the equations given. It is useful for now, nonetheless, to utilize this formalism to describe the kinetic effects of biochemical and pharmacological manipulations of rod cells. The formalism provides, at the very least, a common framework to describe these effects. Unfortunately, this is done rarely in the current literature.

For a fully dark-adapted rod, the peak amplitude of the photocurrent continues to increase with light intensity beyond the linear range up to a saturating value at which the dark current is totally suppressed. This amplitude saturation occurs when only a few hundred photons are absorbed per cell. Further increments in light intensity result in a lengthening of the time the current remains shut off. The initial rate of rise of the photocurrent also increases continuously with light intensity, up to a saturating value when about 10^5 photons have been absorbed per rod.[52] At light intensities below amplitude saturation, the dependence of the peak magnitude of the photocurrent on light intensity is well described by the Michaelis function:

$$I = I_{max} \frac{E}{E + S} \quad \ldots\ldots \qquad (2)$$

where I is the peak response amplitude, I_{max} is the saturating amplitude, E is the energy of the stimulus flash, and S is the energy at which the amplitude reaches half saturation. This function confirms that for dim lights (E less than s) the peak amplitude increases linearly with light intensity.

The Michaelis function fits well the photocurrent data collected in all species tested to date: rat,[52] toad,[38,53] tiger salamander,[42] and Monkeys.[41] For all species, the number of photons necessary to reach half saturation for dark-adapted cells is remarkably similar: 30 absorbed photons for rats and monkeys, 50 absorbed photons in red and green toads rods, and 50 absorbed photons in tiger salamander. The dependence of the Na permeability of the outer segment on light intensity can also be well described by the Michaelis equation.[43] The adequacy of this equation must, again, be accounted for in the mechanisms of phototransduction and this is not yet unequivocally possible. It is interesting to consider, nonetheless, a model that provides a physical basis to justify the Michaelis dependence of photocurrent amplitude on light intensity. This model was first offered by Baylor and Fuortes[212] in cones and discussed further in rods by Cone.[57] This model proposes that light produces a messenger in the cytoplasm of the outer segment in an amount that is linearly

proportional to intensity. Further, this messenger binds reversibly to the Na channels with a 1:1 stoichiometry and according to the law of mass action. The channels with a bound messenger close. If the ionic permeability of the membrane is simply a function of the fraction of channels that remain open, it can be shown that the peak permeability (and current) would exhibit a Michaelis dependence on light intensity, as found experimentally.

Bader et al.[42] originally reported that the Michaelis function could describe the dependence of photocurrent amplitude on light intensity at any point in time, not only at its peak. More detailed examination by Lamb et al.[58] has shown, however, that for time points preceding the peak the dependence of the photocurrent amplitude on light intensity is best described by the function:

$$I = I_{max} (1 - \exp(-kE)) \ldots\ldots \quad (3)$$

where I is the current, I_{max} is the saturating current, k is a proportionality constant, and E is the energy of the stimulus flash. This function implies a steeper dependence of current on light intensity at times preceding the peak of the photoresponse, and suggests the development of a rapid desensitization process that is established by the time the rod reaches its peak photocurrent.

2. Characteristics of the Photovoltage

As pointed out above, the rod membrane potential hyperpolarizes in response to light. Since light reduces the Na permeability of the outer segment, but not the K permeability of the inner segment, the hyperpolarization might simply arise from the behavior of the membrane as a K electrode. Indeed, the rod membrane potential during a saturating light response (when all Na permeability is abolished) behaves approximately, but not exactly like an ideal K electrode.[23,24] This deviation from ideality arises from two principal facts: (1) the membrane shows a finite, if small, permeability to Cl and Ca and (2) the K channels are voltage and time dependent, as discussed in detail below. The membrane hyperpolarization should, in any event, decrease the K efflux from the photoreceptors. Indeed, light-dependent reduction in radioactive K efflux has been measured in frog retinas.[49] Moreover, measurements with ion-selective electrodes in the extracellular space around the inner segment show light-dependent changes in K concentration that can be quantitatively correlated with a reduction in the passive K efflux consequent to membrane hyperpolarization.[59]

Only recently, has it been possible to record simultaneously photovoltages and photocurrents from single, isolated rods. Such technical development required the application of voltage clamp methods to rod cells isolated from the rest of the retinal tissue. Cell isolation can be achieved either by gentle, but extensive mechanical trituration[34,60,61] or by gentle enzymatic digestion of the retina.[42,62] The time course of the photovoltage differs significantly from that of the photocurrent. In the linear range, the photovoltage response is superficially similar to the photocurrent, but it always reaches to a peak preceding the photocurrent peak.[42,63,64] Nonetheless, the time course of these dim light photovoltages can be well fit by the same mathematical expression (Equation 1) that describes the kinetics of dim light photocurrents by selecting slightly faster time constants.[33] In contrast, the current and voltage responses to near saturating lights differ substantially. Most notably, the photovoltage exhibits a transient hyperpolarization that decays as the potential rapidly relaxes to a steady and less negative plateau.[23,33,65,66] This spike-like transient, often referred to as a "nose", is never observed in the photocurrent records. Because the photocurrent measured in the outer segment does not show a "nose", this voltage relaxation must reflect the activity of ionic channels different than the light-sensitive channels located in the outer segment. Indeed, the "nose" can be eliminated by low concentrations of cesium[67] an agent known to block voltage-dependent K channels.[68] The presence in the inner segment of light-insensitive

channels that are time and voltage dependent has been demonstrated directly in voltage clamp studies.[27]

Bader et al.[27] in voltage clamp studies of isolated inner segment of the tiger salamander have identified five currents, each distinct in ion selectivity, activation voltage, time dependence and controls. These are (1) a current carried equally by Na and K ions, with a reversal voltage at −32 mV and blocked by external Cs, (2) a K current with reversal voltage around −70 mV and blocked by tetraethylammonium, (3) a Ca current blocked by Cobalt, (4) a K current that is activated by internal Ca, and (5) an anionic current, principally carried by Cl and activated by internal Ca. It is important to reemphasize then that, as pointed out above, the rod photoreceptor is functionally polarized: the outer segment contains only light-sensitive channels, whereas the inner segment contains a number of voltage- and time-dependent, but light-insensitive channels. The electrical signal generated by the light suppression of the dark current in the outer segment spreads passively to the inner segment. Here the kinetics of the photosignal are speeded up by the activation of the local channels. This increased speed is apparent, for instance, in the fact that the dim light photovoltage reaches to a peak preceding that of the photocurrent and it reflects the removal from the photovoltage signal of its low frequency components, and can be described as representing the action of an electrical high-pass filter.[60,64] This high-pass function of the rod inner segment apparently depends principally on the activity of the Cs-insensitive but voltage-dependent K channels.[69] Unfortunately, not enough information is yet at hand to be able to fully reconstruct the electrical photoresponse of the rods from the properties of its individual membrane channels.

In the retina, rods do not function as independent units, but rather as elements of an electrical network in which signals generated by illumination of one rod spread and produce an electrical response in distant, unilluminated rods.[66,70-73] The signal from one rod spreads radially through the network, and as it does the signal becomes smaller in amplitude and faster in time course.[74] The distance over which the signal decays to 36% of its original amplitude varies between 20 and 60 μm (reviewed in Reference 75). The speeding up of the kinetics of the signal as it spreads through the rod network is due to the activation of the voltage dependent channels of the inner segment (in turtle,[66,74] in tiger salamander,[60] in toad.[64,69] In summary, the voltage response of a rod to illumination in an intact retina is not simply a reflection of the light suppression of the dark current. In addition to the photocurrent, the photovoltage is shaped by the activity of voltage- and time-dependent ionic channels in the inner segment membrane and by the electrotonic spread of signals generated by neighboring rods.

The complex electrical features of the rods and the rod network discussed above impose certain quantitative demands on the analysis of the effects of biochemical or pharmacological manipulations on the membrane potential and photovoltage of rods: (1) if a substance is injected into a single rod in an intact retina, it must be considered that the effect recorded could be distorted both in amplitude and time course because the signal generated by the experimental manipulation dissipates throughout the rod network; (2) if a biochemical manipulation changes the resting potential or the photovoltage of a rod, it must be considered which of the several possible membrane ionic channels has been specifically affected. In studying transduction it is better, if possible, to study the effects of given manipulations on the photocurrent of the outer segment or on the photovoltage of intact cells isolated from the Retina. Also, the ionic channel affected by a given manipulation should be specifically identified if possible.

IV. LIGHT ADAPTATION

Rods can respond to dim flashes presented in the dark and also respond when the flashes are presented against a background light. The photoresponse in the presence of background

light, however, is less sensitive than in the dark.[32,65,76-79] This loss of sensitivity, referred to as "light adaptation", is defined by the fact that, when compared with the dark, a smaller photocurrent is produced by the same light intensity. Of course, when rods are returned to darkness they recover their original sensitivity through a process of "dark adaptation". Light adaptation is itself a very sensitive process: absorption of five to eight photons per sec per cell is sufficient to decrease the light sensitivity of the rod by half.[80,81] With light adaptation not only do photoresponses become smaller in amplitude, they also become faster in time course when compared with those of a dark-adapted cell.[36,65,78,79,81] Light adaptation can speed up the photocurrent by as much as a factor of three. In a light-adapted cell the time course of the linear range photocurrent is still well fit by Equation 1, but the value of the time constant necessary to fit the data is faster than the corresponding value in the dark.

V. THE CHANNELS OF THE OUTER SEGMENT PLASMA MEMBRANE

In this section, some of the biochemical and biophysical features of the light-sensitive channels of the outer segment plasma membrane are discussed.

The electrical resistance of the outer segment plasma membrane in the dark can be calculated to be between 10^3 and 10^4 ohm cm^2, a value typical of most cell membranes. Following bright illumination that totally suppresses the dark current, Baylor and Lamb[82] have measured this resistance to be about 10^6 ohm cm^2. This large increase in resistance is the electrical manifestation of the closure of ionic channels. The very high membrane resistance of the outer segment measured in bright light suggests that when all light-sensitive channels have been closed very few, if any, other channels remain open. For channels of typical conductance, about 10×10^{-12} $ohms^{-1}$, the value of the membrane resistance suggests that on the average perhaps less than ten channels remain open. That is, the outer segment contains very few channels in addition to the light-sensitive ones. This conclusion also implies that any ionic flux in or out of the outer segment that does not occur via the light-dependent channels must utilize a molecular mechanism that is not electrically detectable. This is important since the outer segment plasma membrane supports at least two other ionic fluxes: a light-activated Na/Ca exchanger[83-85] and an inward Ca flux.[84]

A. Spatial Distribution of the Channels

The dark current density is uniform along the entire length of the outer segment.[37,38,53] That is, the plasma membrane surface density of open channels in the dark is constant throughout the outer segment. In strong contrast, the transducing properties of the outer segment are not uniform along its length. Dim flash illumination of the base of the outer segment base produces photocurrents that are larger and faster than those produced by the same illumination of the tip.[38,54] Also, following a bright flash that suppresses the dark current for minutes, the process of cell recovery, measured either as the rate at which the dark current is reestablished or as the rate at which the sensitivity to light is restored, is significantly faster at the base of the outer segment than at its tip.[82] This regional difference cannot arise from differences in Na channel density, as discussed above, nor in differences in photon sensitivity.[54] Since we do not understand the mechanisms of transduction, it is premature to attempt a full explanation of these regional differences. Nonetheless, it must be kept in mind that the tip is "older" than the base and, perhaps of more relevance, that the tip is farther than the base from the source of metabolites that are produced in the inner segment such as high energy nucleotides. Since the availability of these metabolites at the outer segment is probably diffusion limited, it would not be surprising if there exists in the outer segment an intracellular gradient of metabolites. Such gradient might account for the observed regional variation in phototransduction.

B. Number of Channels and their Unitary Conductance

The exact number or the surface density of light-sensitive channels in the outer segment is unknown, but some reasonably informed guesses can now be made. Studies of the electrical noise of the photocurrent in small patches of the rod outer segment membrane in the Gecko give an upper bound on the conductance of the individual channels of 50×10^{-15} ohms^{-1}, by assuming that the unitary (shot) event in the noise is given by the activity of the single channel.[216] In toad rods, analysis of the noise of the dark current indicates a shot event amplitude of 2.5×10^{-15} Å, if each shot were a single channel this would correspond to about 60×10^{-15} ohms^{-1} conductance per channel.[80] Analysis of the light-sensitive component of the voltage noise in turtle rods suggests a conductance of 600×10^{-15} ohms^{-1} per channel.[44] These numbers are in reasonable agreement, particularly if the voltage noise value is taken with some quantitative reservation, since direct current measurements provide more reliable means of estimating conductance than voltage measurements do. In a toad rod outer segment a unitary conductance of 100×10^{-15} ohms^{-1} would correspond to a single channel transport rate of about 2.5×10^4 Na ions/sec in the dark (assuming -40 mV membrane potential). For the observed dark current of about 20 pA, the data indicate that about 5×10^3 channels are open in a single outer segment in the dark, or a density of about 5 channels/μm^2 of plasma membrane. The voltage noise measurements suggest that if the channels simply alternate between open and closed states, the mean open lifetime is 200 to 300 msec.[44] The calculated ion transport rate per channel is slow enough that a "carrier" mechanism of transport would be sufficient to account for it. Whether the light-sensitive channels of the outer segment are mechanistically "carriers" or "pores" cannot now be resolved, nor do we now have any information on the kinetics of the channels. That is, we do not know whether they simply open and close, or whether they exist in other conformational states as well.

C. Ionic Selectivity

The channels of the outer segment are unusual in their ion selectivity features. Ion selectivity has been studied both by testing the effects of ionic substitutions on photocurrents[35,47,87] and photovoltages[23,24-26] and by direct measurement of ionic fluxes either with osmotic techniques[43] or radioactive tracers.[48,49] In normal physiological solutions containing 1 to 3 m*M* Ca, the dark current is suppressed when Na^+ ions are replaced by inorganic cations such as K, Rb, Cs, or monovalent organic cations such as choline or Tris.[35,87] These observations were originally thought to be evidence of high ionic selectivity in the outer segment channels. However, Fain and Lisman[29] suggested that current suppression arose from channel closure due to the intracellular accumulation of Ca ions that results from the impairment of the function of a Na/Ca exchanger following removal of extracellular Na. Indeed, if external Ca is drastically reduced (to 10^{-8} *M*) a completely different behavior is observed: when Na is completely replaced, photovoltages[26,88] and photocurrents[87] of normal polarity but slower time course can still be measured if, and only if, divalent ions such as Mg or Mn are present. If divalent ions are completely removed, then a new class of photovoltages[88] and photocurrents[87] appear that are of polarity opposite to that observed under normal conditions. In the presence of very low concentrations of divalent ions the light-sensitive channels appear equally as permeable to Na as to K, Rb, and Li.[88] Radioactive tracer studies also show that in 10^{-8} *M* Ca Na is not much more permeable than K or Rb.[48] No unequivocal explanation for the change in ion selectivity of the photocurrent from Na to divalent cations as a function of external Ca currently exists. It is not possible to resolve whether there occurs a change in the ion selectivity of the regular light-sensitive channels or whether a new class of divalent channels are unmasked that are not functional under normal conditions.

Torre et al.[89] and Capovilla et al.[88] have found that, remarkably, photovoltages of normal polarity and slower time course can also be recorded in the absence of Na even in the

presence of normal Ca if toad retinas are first incubated with any one of several phosphodiesterase inhibitors such as IBMX, RO 20-1724, papaverine, or theophiline. These photovoltages appear entirely similar to those measured in low external Ca and do require the presence of divalent cations such as Mg or Ba. The data show, then, that either low Ca or inhibition of phosphodiesterase can produce new ionic specificities for the photovoltages and slow down their time course. As pointed out above, such changes could involve either changes in ion selectivity of channels already present in the membrane or an unmasking of different channels. Not knowing a mechanism, it is not reasonable to speculate on just how might low Ca or phosphodiesterase inhibition produce the observed changes. However, it is interesting to note that they may operate through a common mechanism since it is now known (see below) that both of these experimental manipulations increase the concentration of cyclic GMP in the outer segment.

D. Voltage Dependence of Channel Conductance

When the membrane potential of the rod is experimentally changed over the range of -120 to -20 mV (which overlaps with the range of the physiological photovoltages) the amplitude of the photocurrent elicited by a constant light remains essentially constant. Above -20 mV the current decreases in amplitude and it reverses polarity between 0 and $+5$ mV. For more positive membrane potentials the current amplitude increases rapidly and nonlinearly with voltage (outward rectification).[42,60] That the photocurrent amplitude is nearly independent of voltage over the physiological range is most surprising, since the changes in ions driving force associated with changes in membrane potential would be expected to reflect in the current amplitude. The voltage independence of the photocurrent amplitude implies that the conductance of the Na channels is voltage dependent. The form of this conductance-voltage function imposes theoretical limits and demands on models of the molecular features of the channels. Work on such models has only recently begun. Unfortunately, as in other channels, the theoretical models are rarely unique and many choices are possible and acceptable. Bader et al.,[42] for instance, have speculated that the Na channels posses a single energy barrier, asymmetrically placed within the channel and located near its extracellular entrance. Baylor and Nunn,[90] on the other hand, have speculated that Na ions cross the channels as dimers, and not as independent units.

The voltage independence of the photocurrent amplitude over the physiological range insures that the photocurrent measured in the outer segment,[37,38,101] even in the absence of voltage-clamp methods, accurately reflects the number and the rate at which the Na channels are closed by light, not confounded by voltage-dependent effects. This electrical feature of the outer segment channels probably serves an important function because it insures that a given light stimulus will produce the same outer segment current regardless of the membrane polarization. The light-generated current in the outer segment is, therefore, essentially independent of the activity of the channels in the inner segment, since the function of these channels is reflected in changes in cell membrane potential. Such a feature provides a one way communication path in which the photosignal generated by the current in the outer segment travels to the inner segment, but the signal generated by the activity of the channels in the inner segment cannot flow back to affect the outer segment. This lack of feedback possibly avoids undesired electrical instabilities and oscillations.

Although it was originally described that membrane voltage had no effect on the time course of the photocurrent,[42] measurements with higher signal resolution have shown that membrane voltage does have an effect on the kinetics of the photocurrent; membrane depolarization slightly slows the decay of the photocurrent whereas hyperpolarization speeds it up.[90,91] Voltage does not appear to affect the rate of rise of the photocurrent. The mechanisms underlying these effects have not been attributed to a direct control by voltage of the kinetics of the Na channels themselves, but rather to control of a Ca removal mechanism that exists in the plasma membrane of the outer segment (see below).[83-85]

VI. SINGLE PHOTON RESPONSE IN THE RODS

As we have seen, the rods are exquisitely sensitive to light: 30 to 50 absorbed photons are sufficient to reduce the dark current by half. Psychophysical experiments showed over 40 years ago that rods can signal the absorption of a single photon.[1] Electrical responses to single photons can be measured experimentally in individual cells.[41,54,92] In an elegant study, Baylor et al.[92] have shown that in toads the photocurrent at very dim steady lights is not steady, but rather shows quantized jumps each approximately 1 pA in amplitude and each with a time course very similar to that of the photocurrent elicited by very dim flashes. Most importantly, they have also demonstrated that each of these quantal events is probably generated by the photoexcitation of a single rhodopsin molecule. The photocurrent arises from the sum or superposition of the individual quantal events. The response generated by the photoexcitation of a single rhodopsin molecule is often referred to as a single photon response (SPR) and we will do so hereafter. However, it must be remembered that a single absorbed photon, because of the quantum efficiency of bleaching, has only a 70% probability of eliciting a single rhodopsin response.

The measured amplitude of the SPR in toads of about 1 pA is in close agreement with the values previously extrapolated from measurements of ionic permeability in frogs[43] and photovoltages in toads[71] and turtles.[93] In toads, the SPR of 1 pA represents a reduction of about 4% in dark current, in monkeys a measured SPR of about 0.6 pA corresponds to a dark current reduction of 2.5%.[41] In general, in all species tested to date the SPR has an amplitude of approximately 1 to 4% of the dark current. The SPR, therefore, corresponds to a reduction in dark current such that 10^6 to 10^7 Na ions are stopped from entering the outer segment per second.

The time course of the SPR is essentially the same as that of the photocurrent following a dim flash and, therefore, it is well described by Equation 1.[54,92] Interestingly, SPRs can be measured even in the absence of light. In darkness, the outer segment current exhibits discrete events of time course and amplitude essentially identical to that of the light-evoked SPRs.[80] These events occur at a rate that is temperature dependent, and in the toad rod at room temperature they proceed at a rate of about 1 every 50 sec. They most probably arise from spontaneous (thermal) isomerizations of the rhodopsin chromophore. From the rate of these dark SPRs it is possible to calculate that rhodopsin molecules have a half-life of about 2×10^3 years in the dark.

VII. MECHANISMS OF PHOTOTRANSDUCTION: INTERNAL MESSENGERS

For several years now an hypothesis has existed that holds that a diffusable intracellular messenger is necessary to link the photoexcitation of rhodopsin, anchored in the disc membrane, to the closure of Na channels located in the plasma membrane of the outer segment since the disc and plasma membranes are structurally separate. Indeed, the rim of the discs is 100 to 200 Å away from the plasma membrane at their point of closest approach. As we have seen, however, thin fibrous connections exist between the discs and the plasma membrane that bridge this gap.[18] In spite of the mechanical connections there is clear and convincing evidence that no electrical connection exists between the membranes, with the exception of 10 to 30 discs that are continuous with the plasma membrane at the base of the outer segment.[94,95] Also, these connections do not provide passage for the diffusion of small molecules since markers such as Lanthanum,[96] Procion Yellow®,[97] and didansyl cystine[98] do not invade the intradisc space when added to the extracellular medium. The discs can act as osmometers and their permeability properties are different than those of the plasma membrane[10-12,99] indicating that the two membranes are functionally dissimilar. Beyond these now "classical" arguments, three recently described physiological features of the rods are

best explained by assuming the existence of an internal messenger in phototransduction. These features suggest that each excited rhodopsin may generate hundreds of messenger molecules, and they also impose restrictions on the manner in which messengers may operate. These features follow.

A. The Magnitude of the Single Photon Response

In toad rods, analysis of the electrical noise of the dark current reveals that this current arises from the sum of randomly occurring "shot" events, each about 1/400 times the amplitude of the SPR.[80] If each of these "shot" events arises from the opening and closing of a single Na channel (see above), then the data suggests that a single photoexcited rhodopsin molecule can close a maximum of 400 channels during a SPR. Similarly, measurements of the light-sensitive membrane voltage noise in turtle rods, although less reliable than current measurements, can be analyzed to show that the SPR can arise from the closure of about 300 independent channels.[44] The large gain of the rhodopsin signal can be explained by a model in which each rhodopsin can generate a large number of transmitter molecules sufficient to close 300 to 400 individual channels. Moreover, as discussed above, if it is further assumed that the amount of transmitter molecules produced increases in linear proportion with the number of rhodopsin molecules bleached, the experimentally observed Michaelis relationship between photocurrent peak amplitude and light intensity can be accounted for.

B. The Stereotyped Amplitude and Waveform of the Single Photon Response

The amplitude of the SPR, about 1 pA in toad rods, varies little from moment to moment and from cell to cell, the standard deviation is within 20% of the mean.[92] This fact can be well explained if an internal transmitter exists that exhibits Poisson statistics in its behavior. To obtain the observed small variation in SPR amplitude it is required that a single rhodopsin generate over 100 transmitter molecules.[92] The waveform of the SPR also varies remarkably little.[92] If a diffusible transmitter indeed operates in phototransduction, the constancy of the SPR form implies that the radial diffusion of a transmitter from the site of rhodopsin photoexcitation to the plasma membrane cannot be the rate limiting step that determines the kinetics of the photocurrent. This corollary follows from the realization that if the time course of the photocurrent was controlled by the radial diffusion of the messenger, then the SPRs would have different time courses depending on whether they were generated by rhodopsin molecules located near the plasma membrane or near the center of the disc. If radial diffusion away from rhodopsin is not rate limiting, and if one insists on allowing the messenger to control the photocurrent kinetics, two alternative models are worth considering: (1) the transmitter is not produced at the site of rhodopsin excitation, but rather at some fixed site(s) on the disc membrane and the link of rhodopsin to this site is rapid with respect to the time course of the photocurrent or (2) the arrival of the transmitter at the plasma membrane is rapid and the kinetics of channel closure are all controlled by the rate of transmitter removal.

C. The Longitudinal Spread of the Transducing Signal

If an outer segment is illuminated with a narrow slit of light over a restricted portion of its length, Na channels beyond the area of illumination also close. The longitudinal spread of signal, however, is restricted. In rats[37] and toads,[58] the spread has been experimentally shown to be within 12 to 16 μm from the site of illumination, but these values are only upper limits on the true spread because of the limited resolution of the experimental method. Lamb et al.[58] developed a theoretical model to determine a more accurate limit on the signal spread and found it to be about 3 μm. Such spread can, again, be best explained by assuming that transduction involves a messenger that can diffuse away from the area directly illuminated. The effects of light adaptation also spread a small distance away from the site of

illumination along the length of the outer segment.[58,100,101] The limit of the spread of light adaptation is experimentally determined to be about 6 μm in toads[58] and about 5 to 20 μm in frogs.[100] The magnitude of the spread of photoexcitation demands that if a messenger exists, it diffuses along the outer segment with a diffusion constant of about 10^{-7} cm^2/sec. This value is about 100 times slower than the diffusion constant of a small ion in water. The slow diffusion constant can be accommodated in the internal messenger model with one of the following three possibilities: (1) the diffusing messenger is not a small molecule, but one whose Stoke's radius is about 100 times larger than that of an ion; (2) the diffusing molecule is indeed small, but its longitudinal spread in the outer segment is mechanically obstructed by the presence of the discs. Calculations[58,102] show that the mechanical barrier of the discs could reasonably slow diffusion of a small molecule by a factor of 100; (3) the diffusing molecules can bind specifically to immobile sites. For example, McLaughlin and Brown[103] have calculated that if Ca ions were the messengers their diffusion constant in the outer segment cytoplasm could be expected to be 10 to 100 times slower than in water because of Langmuir adsorption to immobile Ca binding sites, such as the negatively charged phospholipids in the disc membranes.

That an internal messenger traveling from disc to plasma membrane provides a good hypothetical model to account for many of the electrophysiological features of transduction does not, of course, either prove the model or strictly eliminate other possible models. The model is worth pursuing at this time because molecules that may play the role of messengers in transduction have been specifically identified. In the next section, the information now available on possible internal messengers is discussed.

VIII. THE SEARCH FOR AN INTERNAL MESSENGER: FUNCTIONAL CRITERIA

The electrophysiological data discussed above impose certain minimum criteria that must be fulfilled by a molecule that acts as an internal messenger in rod phototransduction. In the simplest of models, one that assumes that rhodopsin generates (either by increasing or decreasing concentration) a single class of diffusible messenger to link its photoexcitation to the ion channels in the membrane it would be expected that:

1. The messenger exists in the outer segment and its concentration changes following illumination. This concentration change: (a) must be rapid, certainly no slower than the rate of change of the photocurrent, (b) must be large since one rhodopsin apparently controls hundreds of individual channels, and (c) must change in magnitude as a function of light intensity at least over the same range that the electrophysiological response occurs.
2. The messenger molecule, when provided to the cell, can fully mimic the electrophysiological effects of light. That is, the messenger must specifically control the ion channels of the outer segment and must control not only the amplitude of the current through these channels but also its kinetics.
3. The outer segment must contain the biochemical machinery necessary to control the level of the messenger molecules. Since the concentration change of the messenger must be transient, biochemical mechanisms linked to rhodopsin that control the generation of the messenger and its removal must be identified.

Ca ions and cGMP molecules have been proposed as internal messenger candidates in rod transduction. A messenger role for Ca ions was first proposed by Hagins and Yoshikami.[104,105] In their model, Ca ions are contained by the discs in the dark and the free cytoplasmic concentration is low. Following illumination, a permeability increase of the

disc membrane leads to the passive release of Ca and an increase in its cytoplasmic concentration. Ca controls the membrane channels through reversible binding to specific sites on the channels. The dark, low level of Ca is restored by active transport of Ca into the discs. A messenger role for cGMP has been detailed by several independent investigators.[106-110] Interest in a possible role for cyclic nucleotides was generated by the original observation of Bitensky et al.[111] In this model, cGMP levels in the cytoplasm of the outer segment are high in the dark as maintained by the balance of the activities of the anabolic enzyme guanyl cyclase and the catabolic enzyme, cyclic nucleotide phosphodiesterase. Following illumination, phosphodiesterase is activated and the levels of the nucleotide decrease. This reduction acts as a signal on the ion channels directly or indirectly through a nucleotide-dependent phosphorylation of small proteins.[108,112,128] The high dark levels of cGMP are restored because the activation of the phosphodiesterase is only transient. To guide the discussion of the information now available we will analyze the evidence that addresses whether Ca ions or cGMP molecules meet the minimum criteria of a transduction messenger set forth above. Even though it is true that those criteria evolve from the simplest possible model, they provide a framework from which a discussion of more complicated models may proceed.

A review of the biochemical literature poses a quandry because, in contrast with the electrophysiological studies in which fair experimental agreement exists in the literature, biochemical studies are often in disagreement of facts. These disagreements may reflect a limitation of the experimental methods. In particular, analytical investigations often require large amounts of outer segments which must be isolated from the rest of the rod cells and collected from many retinas. Particular precautions must be taken to preserve the integrity plasma membranes of isolated outer segments prepared in large quantities. It is very possible that in some cases the isolation of outer segment and destruction of the plasma membrane results in the loss of regulatory components, substrates, and co-factors necessary for normal transduction. For instance, several groups have demonstrated that even in freshly isolated outer segment obtained through gentle procedures that preserve the plasma membrane, nucleotides such as ATP and GTP are lost within minutes after isolation, presumably because of the loss of the mitochondrial synthetic machinery of the inner segment.[113-115] Also, the Na permeability of the outer segments can be lost unless precautions are taken to minimize oxidation.[116] Therefore, we will attempt to distinguish between biochemical information collected in whole rod cells, usually intact retinas, and that collected in isolated outer segments or discs. We will hold the view that differences in data between these two types of preparations are important because they inform us of features of the biochemical processes in the cell, but that the physiology of the whole cell is best explained by data collected in intact cells.

IX. EVALUATION OF Ca AS AN INTERNAL MESSENGER

A. Criterion 1

The outer segment should contain Ca and illumination should change the free Ca concentration in the cytoplasm. The outer segment indeed contains a high concentration of total Ca. In the intact rat retina, the concentration of Ca in the outer segment, evaluated with ion microprobe analysis, is about 2.5 mM/ℓ.[35] Atomic absorption spectroscopy shows that isolated outer segments contain, 1.5 to 5 mM/ℓ in frogs[117,118] and from 1 to 10 mM/ℓ total Ca in cattle depending on the method of isolation.[119] The total disruption of the plasma membrane, even in the presence of a Ca chelator, does not significantly reduce the Ca content of outer segments suggesting that most of the Ca is contained within the rod discs.[118] In a frog, each disc contains about 2×10^5 Ca ions, but whether the ions are free or bound is unknown.

Since most of the Ca is contained in the discs the cytoplasmic free concentration in the dark is expected to be low, but its precise value is not known. Typical cells have free Ca cytoplasmic concentrations of 10^{-7} to 10^{-6} M/ℓ. From the titration of Na permeability of the plasma membrane with added Ca, Hagins and Yoshikami[120] in rats and Wormington and Cone[116] in frogs have estimated that the free Ca concentration in the outer segment is less than 10^{-6} M/ℓ in the dark.

Direct evidence is lacking as to whether illumination results in an increase in cytoplasmic free Ca in the outer segment. On the other hand, indirect evidence suggests strongly that such increase indeed occurs. Measurements of Ca concentration in the space immediately outside the outer segment in intact toad[83,84] and rat[85] photoreceptors have shown that illumination leads to the release of Ca from the outer segment. The Ca release is directly linked to the photoexcitation of rhodopsin and it is not secondary to changes in dark current or membrane voltage. Following a dim flash of light the Ca release begins nearly simultaneously with the change in dark current. In dark-adapted toads, the amount and rate of Ca release increases with light intensity over a range that overlaps that of the electrophysiological response. The rate of release increases nearly linearly with light and saturates when about 500 photons are absorbed per rod, the saturating rate of release is about 10^6 Ca ions per rod per second. The amount of Ca released increases linearly with light intensity up to a few hundred absorbed photons per rod and nonlinearly beyond that until it saturates at about 10^5 absorbed photons per cell. In the linear range, each outer segment releases about 2×10^4 Ca ions per photon absorbed in toads and about 500 in rats. It has been argued, without direct proof yet, that this light-dependent release of Ca results from the activation of plasma membrane Na/Ca exchangers caused by the raise in intracellular Ca that follows illumination.[83-85] If this interpretation is correct, the release data is evidence that in intact rod outer segments there occurs a raise in cytoplasmic Ca concentration that is sufficiently large, sufficiently rapid, and overlaps the light intensity range of the physiological response as necessary to satisfy the requirements of an internal messenger.

B. Criterion 2

An increase in cytoplasmic free Ca concentration should fully mimic the electrophysiological effects of light. Increasing the Ca concentration in the medium bathing rods from its normal value of 2 to 3 mM/ℓ to 15 to 20 mM/ℓ specifically suppresses the dark current (in rats,[43] in toads[87]) and the Na permeability of the outer segment membrane[43,116] suggesting that external Ca either reduces the number of open channels in the outer segment or their unitary conductance. Which of the alternative mechanisms actually occurs cannot yet be resolved. The increased extracellular Ca also reduces the amplitude of the photocurrent,[43,87] and because of its effects on the Na channels of the outer segment, increased extracellular Ca also affects the rod membrane potential: Ca hyperpolarizes the membrane potential in the dark and the peak amplituded of the photovoltage is reduced.[23,81,121-123] It is important to note, however, that increased extracellular Ca does not change the kinetics of the photovoltage nor the light sensitivity of the cell.[81,121]

The effects of increments in extracellular Ca are relevant to a discussion of an intracellular messenger role for Ca only if these changes lead to changes in intracellular Ca. Such critical assumption is yet to be directly proven experimentally. However, the dark current in intact rat rods[120] and the Na permeability of isolated outer segments[116] can be totally suppressed by an external Ca concentration of only 0.01 mM/ℓ in the presence of the ionophores X-537 or A-23187 that render the plasma membrane highly permeable to Ca, whereas the same effects require 20 mM/ℓ Ca under normal conditions.[43,47] External Ca is thus about 2000 times more effective in suppressing the dark current when the plasma membrane is rendered highly permeable to Ca than when it is not. Further evidence that Ca acts internally is the observation that intracellular Ca injection into the rod outer segment produces a transient

hyperpolarization of the membrane potential in the dark.[124] A critical consideration in the interpretation of the effects of Ca ionophores is whether they modify only the plasma membrane or other membranes as well. In rat rods, the effects quoted above seem to be true only for about 10 to 20 min following the addition of the ionophore. Longer times produce complicated effects that culminate in the disappearance of the dark current and the photoresponse.[47] In isolated outer segments all experiments are completed within 10 min of the addition of the ionophore.[116] It, thus, appears likely that the effect of the ionophore may be restricted to the plasma membrane only at first, while other membranes, including perhaps the discs, are permeabilized later. If such is the case, the observations on the early effects of Ca ionophores are consistent with the proposition that intracellular Ca is more effective in controlling the Na channels of the outer segment than extracellular Ca. They do not rule out, however, that Ca may also exert some effects from the extracellular surface of the plasma membrane.

Lowering the extracellular Ca concentration also affects the Na channels of the outer segment. Between normal concentrations and 10 $\mu M/\ell$, lowering Ca increases the dark current,[47,87] depolarizes the membrane potential in the dark,[23,122,125,126] and increases the amplitude of both photocurrent[47,87] and photovoltage,[122,125,126] but the light sensitivity of the cell remains essentially unaffected.[125,126] It is interesting to note that over this range of changes in Ca concentration the amplitude of the dim light photocurrents simply scales in proportion to the amplitude of the dark current.[47,87] That is, the fraction of channels blocked by a single photon remains nearly constant despite the increase in the number of channels (or their unitary conductance) produced by lowering the Ca concentration.

In contrast to the effects of extracellular Ca concentrations down to 10 $\mu M/\ell$, concentrations less than 1 $\mu M/\ell$ initially raise the dark current by as much as a factor of 10, but within 20 to 50 sec the current magnitude returns to a value only slightly larger than that in normal Ca.[87,127] This rapid restoration of the dark current most probably reflects changes in the membrane ionic gradients due to a large intracellular Na accumulation expected to follow the sudden and large increase in permeability of the plasma membrane. In the very low external Ca, the amplitude of the photocurrent and photovoltage are also increased but, in contrast with the effects of Ca levels above 10 $\mu M/\ell$, there is also a loss of cell photosensitivity and a slow down of the time course of the photoresponse.[87,122,125,126,129] In toad rods, Bastian and Fain[125] have shown that after incubation in very low Ca for a few minutes the number of photons necessary to half saturate the response amplitude (s in Equation 2) increases from its normal value of about 50 to about 200 in 1 $\mu M/\ell$ Ca and then increases proportionally with the decreasing Ca concentration up to a value of about 50×10^4 photons in 10^{-9} M/ℓ Ca.

In very low external Ca, the peak amplitude of the photovoltage shows a much steeper dependence on light intensity than normal and is well described by the function:[125,126]

$$V = V_{max} \frac{E^n}{E^n + S} \cdots\cdots \quad (4)$$

where V is the peak photovoltage amplitude, V_{max} is the saturating amplitude, E is the energy of the stimulus flash, and n is a parameter whose value is always larger than 1 and increases with the loss of Ca. Prolonged incubation in the very low Ca solutions (over about 15 to 20 min) can lead to total loss of the photoresponse. No unique explanation is yet available for the effects of very low Ca on the photoresponse time course and sensitivity. Very low external Ca must produce effects beyond opening Na channels since cell sensitivity to light is also lost. If changes in Ca metabolism are to fully account for these observations two alternative models can be envisioned. The first one proposes that very low external Ca depletes intracellular Ca stores that would normally be mobilized in response to light. Loss of sensitivity arises because fewer Ca ions are mobilized at a given light intensity and,

therefore, more light is necessary to achieve significant Na channel closure. In the second model, Ca stores are assumed to remain unaffected, but low external concentrations are proposed to strip Ca away from cytoplasmic binding sites that are normally nearly saturated. Loss of sensitivity arises because the Ca ions released by light are bound in part by the stripped sites; the cytoplasm acts as though it had a high buffering power for Ca. This increased buffering power leads to changes in sensitivity and time course.[125] These two possibilities cannot now be fully distinguished.

Buffering intracellular Ca also affects the photoresponse. In rat rods, Hagins and Yoshikami[127] have found that small amounts of intracellular EGTA (5 to 10 $\mu M/\ell$) can reduce the sensitivity of the photocurrent to dim light only when external Ca is low, about $2 \times 10^{-7}\ M/\ell$, but are without effect in the presence of normal external Ca. They interpreted these results as evidence that EGTA buffers a transient Ca concentration increase that acts as a message in transduction. However, this interpretation has been questioned by Kaupp and Schnetkamp[130] since they estimate the normal cytoplasmic Ca buffering to be about 0.1 to 0.2 mM/ℓ, and 10 μM EGTA should, therefore, produce a negligible increase in the buffering power. In view of this criticism, the interpretation of Hagins and Yoshikami must also imply that in very low external Ca the cytoplasmic Ca buffering power of the outer segment is reduced relative to its values in normal Ca. In contrast, when relatively large injections of EGTA are made into toad rods, the amplitude of the membrane photovoltage is attenuated even in normal external Ca.[110,124,131] EGTA injections act through a specific effect on the light-sensitive Na channels.[131,132] Unfortunately, the quantitative effects of EGTA on photovoltages cannot be reliably investigated in these experiments because of possible voltage distortions due to photoreceptor coupling and to effects on inner segment channels. Surprisingly, the effects of EGTA microinjection are transient and disappear shortly after the end of the injection. This observation has not been explained, but may arise from loss of EGTA from the injected cell either across the plasma membrane or to neighboring cells through cell-cell junctions.

While raising intracellular Ca does control the amplitude of the photocurrent, Ca does not fully mimic the electrophysiological effects of light. In particular, in toad rods an increase in the external Ca concentration does not accelerate the time course of the photovoltage nor does it significantly decrease the cell sensitivity to light.[81] Such changes in kinetics and sensitivity are always observed in the presence of background lights.[38,65,81] These observations may indicate that adaptation is served by mechanisms different than those underlying excitation. In any event, changes in Ca concentration alone are not sufficient to account for the full range of the electrophysiological changes in response to light.

C. Criterion 3

The outer segment should contain the biochemical machinery linked to rhodopsin necessary to control the cytoplasmic levels of Ca. Evidence to satisfy this criterion is particularly weak. The estimates from electrophysiological data and the measurement on light-dependent Ca release from the outer segment demand that following a brief dim flash, the cytoplasmic Ca concentration increase at a rate of 10^3 to 10^4 Ca ions per photon per second. The critical measurements of cytoplasmic Ca concentration in outer segments of intact retinas has so far not been technically feasible. On the other hand, extensive and repeated efforts to measure light-dependent Ca fluxes in isolated disc membranes have failed to reveal fluxes of the expected magnitude. In general, in reports published up to early 1983 the various disc membrane preparations used, ranging from freshly isolated and unpurified to frozen and extensively purified material, combined with various Ca detection techniques including radioactive tracers, indicator dyes, and ion-selective electrodes yielded results indicating either no light-dependent Ca release or release that is too small and too slow, 1/70 to 5 Ca ions per photon per second. The reader is referred to Noll et al.[133] and Kaupp and Schnetkamp[130]

for a thorough collection of these data. There does seem to exist a very rapid Ca release that is small (0.5 to 1 Ca per photon per 20 msec) and is associated with changes in Ca binding to the disc membranes.[134,135]

The failure to observe Ca release from discs in vitro consistent with in vivo observations suggested either that isolation and manipulation of disk membranes results in loss of components critical to the release of Ca or that the light-regulated Ca stores are not sequestered in the disc but are in the cytoplasm.[136] In support of the first proposition it can be pointed out that 10 to 15% of the protein in the disc membranes are peripheral and can be released by changes in medium composition or ionic strength.[137-139] It is particularly interesting that George and Hagins[140] have recently reported that frog discs freshly isolated, not washed and suspended at high concentration in a small volume of composition similar to that presumed for intracellular space, show a large light-dependent Ca release. In the best of cases nearly all the Ca contained by a single disc can be released in about 90 sec when only a few rhodopsin molecules are bleached. The release rate, however, while faster than any previously reported, is still slow relative to the rate demanded by photocurrent and Ca release measurements. For instance, at light levels of 0.15 photons per disc, the Ca efflux rate increases slowly and takes about 15 sec to reach a peak value of about 5×10^2 Ca per disc per second. In contrast, at this bright light levels the photocurrent reaches saturation in less than 0.1 sec and the Ca is released immediately at maintained rates of about 5×10^3 Ca per disc per second. Clearly, discs are complex structures and great care must be taken to develop conditions in which their physiological properties are comparable to those in vivo.

The mechanisms that might restore Ca levels to their dark values are poorly understood. The Na/Ca exchanger in the plasma membrane removes the Ca from the cytoplasm, but mechanisms must also exist to restore it to its light-sensitive storage sites. There does not exist good experimental evidence that isolated discs can actively transport Ca with the stoichiometries and kinetics required to match the electrophysiological data. Isolated discs in the presence of high GTP/GDP ratios exhibit a cGMP dependent Ca uptake.[140] In intact rod cells, the transport of Ca into the discs is reported to be very slow, less than 5 Ca ions per disc per second and light insensitive.[136,211] Illumination of isolated frog outer segments leads to a slow hydrolysis of GTP, with a half-time of several seconds.[113,114,141] The claim has been made that this may represent the consumption of metabolic fuel associated with active sequestration of Ca. While such explanation is possible there is no proof that it is correct. If Ca is an internal messenger, clearly the biochemical mechanisms that control its release and reuptake into storage sites are yet to be fully specified.

In summary, Ca ions meet several of the minimum criteria demanded in a simple model of an internal messenger, but do fail in two important points: (1) increased Ca levels do not mimic in full the effect of light on the kinetics and light sensitivity of the photoresponse, and (2) changes in intracellular Ca following illumination have only been proven indirectly. If such changes occur, the biochemical processes that bring them about are largely unknown.

X. EVALUATION OF cGMP AS AN INTERNAL MESSENGER

A. Criterion 1

The outer segments should contain cGMP and illumination should change the free cGMP concentration in the cytoplasm. The outer segment indeed contains a high concentration of cGMP. The cGMP level has been measured directly in outer segments either freshly isolated[142] or microdissected away from rapidly frozen retinas.[143-145] In normal physiological solutions, dark-adapted frog outer segment contain about 45 pM of cGMP per mg dry weight. This corresponds to be about 50 $\mu M/\ell$ or about 0.015 cGMP molecules per rhodopsin or about 3×10^7 cGMP molecules per outer segment. The photoreceptor layer contains 70 to 90% of all cGMP in the retina[146-148] and the outer segments alone contain about 50 to 60%.[143-145]

Measurements of modulated changes in cyclic nucleotide levels have traditionally been limited to determinations of steady-state concentration of the nucleotides in tissue, assisted by in vitro measurements of the activities of the anabolic (cyclase) and catabolic (phosphodiesterase) enzymes that control the cyclic nucleotides. If changes in the dynamics of the nucleotide metabolism occur it had been impossible, until very recently, to measure them.[149] Our understanding of the role of cGMP in photoreceptor function has suffered because of these technical limitations. In particular, there are remarkable differences in the data reported in studies of isolated outer segments when compared with data obtained in whole rod cells. In isolated rod outer segments illumination can decrease the levels of cGMP by up to 50%[109,142,150] but it is also possible to find conditions under which no changes in cGMP concentration are observed.[151] In a detailed study of isolated frog outer segments suspended in low external Ca, Woodruff and Bownds[142] found that bright, steady illumination (10^5 photons per rod second) reduces cGMP levels with a half-time of 100 to 200 msec, corresponding to a rate of about 6×10^7 cGMP molecules disappearing per outer segment per second. The magnitude of the cGMP decrease is proportional to the logarithm of light intensity and Woodruff and Bownds have extrapolated from their data that a single photon can lead to the disappearance of 10^4 to 10^6 cGMP molecules.

There is general agreement that in intact retinas continuous bright illumination can also decrease the levels of cGMP by up to 40 to 70%.[143,144,146,147,152-157] It is often difficult to compare many of the results in the literature because the reports do not specify the light intensities tested or the time course of the changes. On the other hand, Killbride and Ebrey[158] have carefully measured the time course and the light-intensity dependence of the cGMP loss in intact frog retinas following illumination. They could not detect any changes in cGMP concentration during up to 30 sec of continuous dim light. At moderate light levels (70 to 700 photons per rod sec) small changes in concentration starting many seconds after the initiation of illumination were observed. At the brightest light intensities tested (about 10^7 photons per rod second), intensities that would totally suppress the dark current in under 0.1 sec, the cGMP level began to decrease with a delay of 3 to 5 sec relative to light presentation. Experiments such as these in whole retinas can be criticized because, although photoreceptors may contain up to 60% of all cGMP[143-145] many cell types are sampled simultaneously. These objections were addressed by Govardovskii and Berman,[145] who specifically measured the cGMP content of outer segments separated by microdissection from retinas rapidly frozen following illumination. Again, they found that dim flashes just sufficient to saturate the photocurrent amplitude (about 2000 photons per rod) did not change the cGMP concentration. Bright steady illumination, on the other hand, did reduce the cGMP levels following a delay of about 3 sec.

The disparity between data obtained in different preparations of isolated outer segments and those obtained in intact cells can probably be resolved by considering the important recent experiments of Goldberg et al.[159] Using an ingenious method they have shown in the rabbit retina that the effect of dim and moderate lights is to increase the turnover rate of the cGMP metabolic pathway without changing the steady-state concentration of cGMP. Thus, in an intact cell dim and moderate lights apparently activate both the guanylate cyclase and the phosphodiesterase to approximately the same extent, thereby maintaining a nearly constant level of the guanyl nucleotide in the presence of an accelerated flux of the nucleotide through its metabolic cycle. In isolated outer segment, in contrast, one may infer that the balance between anabolic and catabolic enzyme activities can be perturbed. For instance, it is possible that the guanylate cyclase activity is reduced in isolated outer segments and unable to overcome the effects of the light-activated phosphodiesterase known to be fully active in the isolated membranes.[151,160-163] The possibility of a loss of cyclase activity is reasonable since its substrate, GTP, has been shown to be rapidly lost upon outer segment isolation.[113,114,141]

B. Criterion 2

A change in cytoplasmic cGMP concentration should fully mimic the electrophysiological effects of light. Data on the electrophysiological effects of cGMP are technically difficult to obtain and have only recently begun to be gathered. To date, most of the information available is limited to studies of effects of the nucleotide on membrane voltage in the dark and following illumination. Analysis of the mechanisms underlying effects of cGMP on rod membrane potential and photovoltages must be cautious because: (1) as discussed above, photovoltage effects recorded in a single rod in the retina can be distorted in amplitude and waveform by the electrical coupling of the rod to its neighbors and by the activity of the voltage sensitive channels in the inner segment. (2) Brown and Pinto[164] have recently shown that in isolated intact rod cells intracellular injections of cGMP in the dark generates a membrane current whose reversal potential is different than that of the photocurrent. This suggests that cGMP, in addition to controlling light-sensitive channels[132] may alter the ion selectivity of these channels and/or it may control other channels in the rod.

The intracellular levels of cGMP in rods have been experimentally modified by three alternative methods: (1) injection of the nucleotide, (2) pharmacological inhibition of the phosphodiesterase, and (3) intracellular injection of purified molecular components, typically proteins, that regulate the metabolism of cGMP in the outer segment. The functional effects of intracellular injection of cGMP apparently depend strongly on the characteristics of the injection. If the injections produce only transient depolarizations of the membrane potential in the dark, then illumination following the injection produces photovoltages that, when compared to normal responses, are larger in amplitude and exhibit a prolonged latency between the light flash and the initiation of the voltage change. However, the waveform of the photovoltage appears to be the same as that of the normal response.[110,165-167] The magnitude of the latency change in the photovoltage depends on the intensity of light; the latency shortens as the intensity increases.[167] Interestingly, the transient depolarizations produced by cGMP injection in the presence of light are faster in time course than those observed following injections in the dark.[165] On the other hand, if the cGMP injection produces a permanent, rather than a transient, depolarization in the dark, the photovoltages after nucleotide injection are again larger in amplitude but they do not show any latency changes. Moreover, their time course is now slower than normal. The light sensitivity of the cGMP injected cells is not different than that of uninjected cells.[126,129]

Inhibitors of cyclic nucleotide phosphodiesterase, most notably isobutylmethylxanthine (IBMX) have been used to alter the metabolism of cGMP and to test the consequence of this alteration on the electrical photoresponse of the rods. The interpretation of the data reported to date in terms of molecular mechanisms must be cautious because, in addition to the limitations of voltage measurements discussed previously, the specificity of IBMX on the rod phosphodiesterase is unknown. It is surprising, for instance, that while incubation with IBMX does increase the level of cGMP in the dark it does not inhibit the bright light induced decline in cGMP level.[154] Extended incubation (over 10 min) of toad retinas in IBMX in the dark leads to membrane depolarization. In IBMX the photovoltages are larger in amplitude and slower in time course than normal.[126,129,168,169] It is particularly interesting that IBMX treatment increases the light sensitivity of the cell. Such increase is apparent in that less light intensity is necessary to reach half of the response amplitude (s in Equation 2).[126,129,169]

In a careful study, Capovilla et al.[169] have investigated the effects of IBMX on the photovoltages elicited by dim flashes. Limiting investigations to this range of intensities, where voltage excursions are small, decreases the uncertainties introduced by the action of voltage-dependent channel of the inner segment. Moreover, IBMX is likely to affect all cells in the retinal rod network and, therefore, kinetic distortions that arise form signal spread between unequal cells are less likely to be present. The time course of the photovoltages in

IBMX-treated cell are slower than in normal cells but can nonetheless be well fit by the same function used with normal cells (Equation 1) by simply selecting a slower time constant. The extent of the response slowdown is quantitatively dependent on the IBMX concentration. As pointed out above, IBMX treatment enhances the light sensitivity of the cell to light and the dependence of the peak amplitude of the photovoltage on light is supralinear and well described by the function:

$$V = C_1E + C_2E^2 + C_3E^3 + ... + C_nE^n .... \quad (5)$$

where V is peak photovoltage amplitude, E is the energy of the stimulus flash, and C1,C2 . . . Cn are proportionality constants; n has a value between 3 and 5 for all cells.

Capovilla et al.[169,170] noted that in the continuing presence of IBMX a steady, dim light background can reverse the electrophysiological effects of IBMX. Thus, the photovoltages become faster in time course, smaller in amplitude, and are no longer supralinear with light intensity. Light opposes the effects of IBMX quantitatively, for example, about eight photons per rod second oppose the effect of 5 $\mu M/\ell$ IBMX while about 80 photons per rod second are required to fully annul the effects of 50 $\mu M/\ell$ IBMX. Light also opposes the effects of raising intracellular cGMP by microinjection.[110,165] While a molecular understanding of these observations will require further work, including unequivocal electrophysiological studies and an exact analysis of the biochemical effects of IBMX, it is nonetheless clear that affecting the metabolism of cGMP produces important changes in the kinetics and the light sensitivity of the rod photovoltage. This is particularly noteworthy because, as pointed out above, increasing the Ca ion concentration does not affect either the sensitivity or the kinetics of the photovoltages, only their amplitude.

In an interesting technical development that should yield important result in the future, Clack et al.[171] have found that intracellular microinjection of some of the proteins involved in cGMP metabolism in the outer segment can specifically affect the rod membrane potential in the dark. Only when specific biochemical effects are correlated with specific electrophysiological consequences will we be able to develop a complete molecular understanding of the effects of perturbations of the metabolism of cGMP.

In intact rods, the steady-state intracellular concentration of cGMP does not appear to control directly the ionic permeability of the outer segment plasma membrane. For instance, progressively lowering external Ca concentration from its normal value down to 10 $\mu M/\ell$ continuously increases the dark current and the Na permeability of the outer segments by up to a factor of 10, yet, over the same range the intracellular cGMP concentration remains constant.[156] Similarly, increasing external Ca concentration to 20 mM/ℓ totally suppresses the dark current and the Na permeability of the outer segments without changing the cGMP steady-state concentration.[156] On the other hand, increasing the extracellular concentration of bicarbonate anions reduces the intracellular level of cGMP without changing the electrophysiological response of the rods.[172,173]

C. Criterion 3

The outer segment should contain the biochemical machinery linked to rhodopsin necessary to control the cytoplasmic levels of cGMP. Both the anabolic enzyme, guanylate cyclase, that synthesis cGMP from GTP and the catabolic enzyme, cyclic 3′-5′ nucleotide phosphodiesterase, that converts cGMP into GMP are found in large amounts in the outer segment. The enzymatic activities and the cGMP metabolism are under sophisticated kinetic control and are exquisitely sensitive to light. The salient details of the information now available are reviewed here, but a thorough review of this enzymology is beyond the scope of this chapter and the interested reader is referred to excellent recent publications.[174-176]

1. Guanylate Cyclase

In the outer segments of frogs and cattle a guanylate cyclase activity of about 1 to 3 n*M*/mg protein per minute has been reported.[177-181] In a frog outer segment this activity can be calculated to yield a rate of about 2 to 6 $\times$ 10^6 cGMP molecules synthesized per outer segment per second. The enzyme is found throughout the outer segment, but apparently its concentration is higher near the base of the outer segment than at the tip.[181] The enzyme is not free in the cytoplasm, and detergents are needed to partially release it from its binding sites.[182,183] Subcellular fractionation studies show that as much as 90% of the enzyme may be associated with the axonemes of the rod;[183,184] these structures include the basal bodies in the nonmotile cilium that connects the outer and inner segments, the array of microtubules at the cillium, and the microtubular structure that extends almost the full length of the outer segment and that lies within the incisures of the discs.[15]

The effects of light on the activity of this enzyme are controversial. In isolated bovine outer segments, bright light is in some instances reported to inhibit the enzyme.[151,178] and in others to produce no change in activity.[185] In intact frog retinas, bright illumination is reported to enhance enzyme activity.[144] This may, again, reflect a difference in biochemical control in isolated outer segments when compared with intact cells. In any event, detailed studies of the time course and light sensitivity of the light effects on this enzyme are now lacking. Nonetheless, the observed increase in cGMP metabolic turnover rate following illumination in intact retinas[159] argues strongly that illumination must enhance the activity of the guanylate cyclase.

2. The cGMP Phosphodiesterase (PDE)

PDE is bound to the cytoplasmic surface of disc membranes and can be removed only by extensive washings with low ionic strength medium.[22,137-139,186] Each disc contains 1 PDE molecule per 60 to 100 rhodopsin molecules or a surface density of 300 to 500 PDE per micrometer[2,139,186] and the presence of rhodopsin is necessary for PDE to bind to membranes.[187] In isolated disc membranes from frogs or cattle, the activity of PDE is low in the dark and is activated 30- to 40-fold following bright illumination; the activity of PDE strictly requires the presence of GTP.[160-163,186,188,189] Liebman and Pugh[107,190] have carried out a careful and elegant kinetic analysis of the features of the light activation of PDE in isolated frog and cattle discs. They have found that the light activation of the enzyme specifically requires low levels of GTP, but high levels of GTP and also ATP inhibit it. In the presence of GTP and no ATP, Liebman and Pugh find that a bright flash that excites about 10^4 rhodopsin molecules per frog disc (a frog disc contains about 2 $\times$ 10^6 rhodopsin molecules) results in a very rapid (under a few hundred milliseconds) maximum activation of PDE that yields activities 30- to 40-fold higher than those in darkness. Weaker flashes produce an increase in enzyme velocity after a time delay; the duration of this delay is inversely proportional to the enzyme velocity. For very dim flashes, those bleaching 10 to 100 rhodopsin per disc, there is a long delay of many seconds between the flash and the enzyme activation. However once the enzyme is fully activated its rate is about 10^6 cGMP molecules hydrolyzed per rhodopsin per second. Since the enzymatic activity per PDE molecule is, at most, 2000 cGMP hydrolyzed per second,[186] the data shows that one rhodopsin must be able to activate at least 500 different PDE molecules. It is important to recognize that the activation of the 500 or so PDE molecules is not instantaneous, but follows a time course approximated by the function:

$$a(t) = a_0\left(1 - \exp\left(-\frac{t}{\tau}\right)\right) \ldots\ldots \quad (6)$$

where a(t) is the activation as a function of time, a_0 is the maximum activation, and tau is a time constant of about 2.5 sec.

The exponential delay of several seconds necessary to achieve maximum activation of PDE by a rhodopsin molecules has been interpreted by Liebman and Pugh to arise from the time necessary for the activated rhodopsin to diffuse on the surface of the disc membrane over an area sufficient to encounter the necessary number of PDE molecules. A single photoexcited rhodopsin must sweep an area of approximately 1 to 3 μm^2 to encounter 500 PDEs. The experimentally observed delay can be quantitatively accounted for in a model that assumes that photoexcited rhodopsin and PDE move on the surface with a diffusion constant of about 1 μm^2/sec. Indeed, rhodopsin is known to diffuse laterally in the disc membrane with approximately this diffusion constant.[191,192] The ability of photoexcited rhodopsin to activate PDE must have a finite lifetime; otherwise, there would be no time limit to the photoresponse. Liebman and Pugh[190] proposed that the well-known ATP-dependent phosphorylation of rhodopsin[193-195,213-215] is the termination signal that renders rhodopsin unable to further activate PDE.[196,197]

3. GTP-Binding Protein (G Protein)

The activation of up to 500 PDE molecules in 2 to 5 sec by a single rhodopsin does not occur by direct interaction of rhodopsin and PDE; a third molecule is involved. This molecule, known as GTP-Binding Protein (G protein) also exists bound to the cytoplasmic surface of the disc membrane and can also be removed by low ionic strength washes.[137-139,186] Each disc contains 1 G protein per 10 to 15 rhodopsin molecules or a surface density of 2000 to 3000 G molecules per μm^2.[22] Isolated disc membranes in the dark, contain tightly bound GDP and illumination results in a GDP for GTP exchange.[114,198] This exchange occurs on the G protein and it is highly amplified.[198] One photoexcited rhodopsin interacts sequentially with several hundred different G molecules[199,200] and catalyzes the exchange of a total of about 500 GDPs for GTPs.[198] The GDP-GTP exchange requires the presence of photoexcited rhodopsin but not PDE and, in turn, the G protein-GTP complex can activate phosphodiesterase in the total absence of excited rhodopsin.[201] Control of PDE, therefore, occurs in two distinct steps: rhodopsin catalyzes the exchange of GDP for GTP in the G protein, and this complex in sequence activates PDE.[176,201] The G-protein-GTP complex directly activates the PDE molecule, apparently by removing from PDE a small 11,000 MW inhibitory subunit.[202,203] The metarhodopsin II photointermediate of rhodopsin is probably the one that interacts directly with the G protein.[204,205] The G protein-GTP complex ceases to activate PDE because of the hydrolysis of the bound GTP to GDP. This GTPase activity is light activated and it is a functional property of the G protein complex itself.[137,206,207]

The disc membranes also exhibit a light-activated rapid and amplified ADP for ATP exchange.[115] The possible significance of this reaction is yet to be determined.

The precise kinetic features in the intact rod of the sophisticated light control of phosphodiesterase briefly discussed above to date and measured only in isolated disc membranes remains to be studied. As discussed before, Goldberg et al.[159] have found that illumination increases the metabolic turnover rate of cGMP to an extent that is dependent on light intensity without any changes in the cGMP steady-state concentration. In the dark, the cGMP pool is calculated to turn over with a half-time of about 1.5 sec. At the highest light level tested, corresponding to hundreds to thousands of photons delivered per rod, the cGMP hydrolysis rate was calculated to have increased by as much as a factor of 125, corresponding to a half-time for the turnover of the cGMP pool of about 15 msec.

In summary, cGMP as a molecule does not meet the criteria of an internal messenger in rod phototransduction. Most particularly, it does not change in concentration in the time course or over the light intensity range in which the electrophysiological responses occur. Moreover, the magnitude of the permeability of the light-sensitive channels is independent of the cGMP concentration in the rod. On the other hand, light produces large changes in the metabolism of this molecule and experimentally induced changes in the metabolism

produce large effects on the kinetics and sensitivity of the photoresponse. These facts strongly suggest that the metabolism of cGMP must play an important regulatory role even if cGMP itself is not the messenger that controls the Na channels of the outer segment.

XI. SUMMARY AND NEW PERSPECTIVES: THE INTERACTION OF Ca AND cGMP

The most superficial statement that can be made in considering the data reviewed above is that neither Ca ions nor cGMP molecules alone meet all the criteria required in the simplest model of an internal messenger in phototransduction in rods. On the other hand, the data strongly indicates that Ca ions, but not cGMP molecules, are likely to act as diffusible messengers whose free cytoplasmic concentration is modulated by light and controls the closing rate of the outer segment membrane channels. Of course, even if the Ca concentration changes with light, the manner in which this concentration change comes about and the mechanisms whereby it controls the channels in the plasma membrane remain a matter of speculation. Indeed, understanding the details of the mechanisms of Ca homeostasis in the dark and the light will likely be a topic of high priority in the research of the near future. Also, studies of the kinetics and the sensitivity of the light-sensitive channels to Ca will surely be subjects of active research both electrophysiologically, through the advent of patch-electrode methods and biochemically, through the development of methods of plasma membrane isolation and purification.

If changes in the cytoplasmic concentration of Ca control the opening and closing of Na channels in the plasma membrane of the outer segment, the precise mechanisms that determine the stereotyped time course of the photocurrent remain unexplained. The time course of the decay phase of the photocurrent is claimed to depend on the removal of Ca from the cytoplasmic space by Na/Ca exchangers in the plasma membrane.[90,91] However, changes in the Ca ion concentration alone may not be responsible for the control of the photocurrent time course; most particularly Ca concentration changes are not responsible for the changes in waveform kinetics associated with light adaptation.[81] The metabolic turnover of cGMP, which increases dramatically with illumination,[159] if not the cGMP level itself, may play a role controlling the kinetics and amplitude of the Ca concentration changes. This contention may be justified by the observations that: (1) changes in the metabolism of cGMP, produced by injecting cGMP or interfering with the phosphodiesterase activity, result in changes both in the light sensitivity and in the time course of the photoresponse[126,169] and (2) changes in the steady-state level of cGMP, produced by very low external Ca concentrations, under 1 $\mu M/\ell$,[154,156,208] also result in a loss of light sensitivity and a slowdown of the time course of the photoresponse.[87,125] The metabolism of cGMP could control the kinetics and amplitude of Ca concentration changes through effects either on the release of Ca from its storage sites or on its subsequent removal from the cytoplasm.

That the Ca levels in the cytoplasm of the outer segment must be under extraordinary dynamic control both in their amplitude and time course of change is particularly exemplified by the fact that the amplitude of the dim light photocurrent scales proportionately with the absolute magnitude of the dark current.[47,87] That is, transduction appears to operate with a variable gain in the diffusible messenger that insures that, at dim light levels, the fraction of the dark current transiently blocked by each photon is constant. Such functional feature could be achieved, for example, if the Ca release mechanisms operated with a feedback control that allowed the cytoplasmic Ca concentration to raise as required to obtain a desired ratio of channels closed over channels open.

We would like to propose that the cGMP metabolism operates as the servocontrol of the Ca concentration change. Several experiments have reported a link between the levels of cGMP and Ca. For example, the steady-state cytoplasmic level of cGMP in the dark is a

function of the external Ca concentration[154,156,208] and in the presence of high GTP concentrations, isolated disc membranes exhibit a cGMP-dependent uptake of Ca ions.[140] Also there exists a competitive interaction in the binding of Ca and cGMP to isolated disc membranes.[209] An intriguing proposition has recently been made by Mueller and Pugh.[210] They have observed that changes in intracellular pH affect the amplitude of the photocurrent but only in the presence of external Ca. Based on these observations they have proposed that protons produced by the light-dependent enhanced cGMP metabolism in the outer segment act as a signal to release Ca from the disc membranes. Their experimental data, however, cannot resolve whether the effects of protons are exerted on the release or on the uptake mechanisms of Ca. Again, the important notion may be that the Ca ions are the diffusible messengers in rod phototransduction, but their metabolism (release and/or uptake) are controlled, at least in part by cGMP metabolism as part of a servocontrol system.

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