Biochemical Correlates of Adaptation Processes in Isolated Frog Photoreceptor Membranes

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ABSTRACT Frog rod outer segments isolated in suspension can maintain much of their in vivo activity. This observation provides us with a simpler system than the intact retina for correlating biochemical and physiological changes. The relevant physiological process, a decrease of sodium permeability by illumination, is assayed as light suppression of outer segment swelling in a modified Ringer's solution. We report here that this decrease is observed over approximately 4 log units of input light intensity and varies with the logarithm of intensity at light levels which bleach between $5 \cdot 10^2$ and $5 \cdot 10^4$ rhodopsin molecules/outer segment-second. In this illumination range responsiveness to light decreases as intensity increases. This sensitivity control system may be linked to light-activated rhodopsin phosphorylation, for inhibitors of this reaction increase light sensitivity. The presence of a second system, which controls the maximum amplitude of in vitro response to light, is revealed in experiments with cyclic nucleotide phosphodiesterase inhibitors. Papaverine addition raises intracellular cyclic GMP (guanosine monophosphate) levels and increases the magnitude of the dark permeability, but does not have a large influence on the amount of illumination required for suppression of this permeability. The data suggest that sensitivity and amplitude, as they are expressed in this in vitro system, are regulated by pharmacologically distinct pathways which use two different light-sensitive enzyme systems.

INTRODUCTION

This paper presents experiments in which we study vertebrate photoreceptor membrane function under conditions allowing simultaneous assay of physiological and biochemical changes. Through such an approach we hope to correlate physiological changes with the molecular events that generate and regulate them. We have previously described an "in vitro assay" for photoreceptor membrane function which makes these experiments possible (1, 3). The assay derives from the work of Korenbrot and Cone on osmotic properties of isolated outer segments (4, 5), and makes use of our further observation that light suppresses slow spontaneous swelling of frog rod outer segments shaken from a retina in the dark (1). Illumination which bleaches only 500 rhodopsin molecules/outer segment-second suppresses swelling if sodium is the major cation present (1). The swelling appears to be regulated by the sodium permeability

mechanism of the outer segment plasma membrane: high sodium permeability in the dark permits sodium chloride entry and swelling; light suppression of this permeability slows swelling (1). By monitoring the volume changes in suspended outer segments we thus have a crude assay of the light-sensitive sodium permeability mechanism.

In these experiments we are not attempting to describe the "in vitro physiology" of isolated outer segments with the same precision and time resolution obtained in electrophysiological studies of intact retinas, and we further assume that some degenerative changes are taking place after detachment of outer segments from the retina. We are able, however, to localize pharmacological effects and chemical changes in the outer segments themselves. Confining our biochemical studies to intact functioning outer segments, we do not control substrate access to enzymes, nor the size of endogenous metabolite pools. Solving these problems by using frozen, lysed, or fractionated outer segments, however, has the obvious drawback of destroying the physiological activity. Although the conditions that we used to study physiology and biochemistry together are less than optimal for studying either by itself, they do enable us to find correlations which would have been difficult to establish using other techniques.

Two recently discovered light-sensitive enzyme systems form a background for this work. This laboratory and others reported several years ago that illumination of photoreceptor membranes can initiate a phosphorylation reaction (6, 7); this reaction is now being studied by several groups (8–10). The terminal phosphate of adenosine triphosphate (ATP) can be transferred either to rhodopsin or to its photodissociation product, opsin (11). The reaction occurs in living frogs (12) and is reversible (11, 12). At low levels of illumination, 30–50 molecules of phosphate are bound to the photoreceptor membrane for every rhodopsin molecule bleached (3, 6). This is a striking result, for small amounts of illumination are having a large effect. We know that bleaching small amounts of rhodopsin also has a large effect on physiology, making the system less sensitive to further illumination (13). One possibility then is that the phosphorylation reaction functions in this desensitization. The experiments of the first part of the Results section are sensitivity measurements which provide the background for a test of this hypothesis, and experiments demonstrating a correlation between inhibition of rhodopsin phosphorylation and increases in light sensitivity are given in reference 2 and described in the discussion which follows.

The second reaction of interest is the light activation of a cyclic guanosine monophosphate (GMP) phosphodiesterase (14–16, Woodruff et al., in preparation). Both we and Bitensky's group have found the enzyme very sensitive to illumination, for bleaching 0.05–0.5% of the rhodopsin present causes its half-maximal activation. A link between this enzyme and outer segment physiology was suggested by our initial observation that papaverine, a cyclic nucleotide phosphodiesterase inhibitor, apparently increases the dark permeability available for suppression by light (1). The second part of the Results section explores this relationship in more detail.

MATERIALS AND METHODS

Each experiment starts as outer segments are gently shaken in dim red light from two dark-adapted bullfrog retinas into 1 ml of a modified Ringer's solution containing 115 mM NaCl, 2 mM KCl, 10 mM HEPES (*N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid), 10% (vol/vol) calf serum, 3 mM EGTA (ethylene glycol bis (β-aminoethyl ether) *N,N'*-tetraacetic acid), 0.5 mM $MgCl_2$, 3 mM dithiothreitol, and 10^{-4} M papaverine. (This solution is used for the experiments of Figs. 1 and 2. It contains the additions which we have found to maximize volume differences between dark and illuminated outer segments [1]. In the experiments of Figs. 3–5, papaverine is added as indicated in the figure legends.) The resulting suspension contains approximately 10^6 outer segments and outer segment fragments. To examine in vitro physiology several 100-μl portions of this suspension are diluted to 15 ml with the same modified Ringer's solution. In a typical experiment of the sort shown in Fig. 1, one of these dilutions is left dark, a second is illuminated continuously with low levels of light, and a third is given saturating illumination (5×10^5 rhodopsin molecules bleached/outer segment-second). Within 5–10 min after the outer segments are shaken from the retina each of these suspensions is being periodically sampled with a particle-sizing apparatus (described in reference 1) to determine the average volume of the outer segments present. The data are plotted as shown in Fig. 1. Dark outer segments increase in volume most rapidly; illuminated outer segments swell more slowly. At the lower light intensities, swelling suppression persists as long as illumination continues, and more rapid dark swelling returns when the light is turned off (cf. [1]). In all of the experiments of this paper continuous illumination is used.

For illumination of outer segment suspensions light from an Osram 12-V halogen lamp (no. 64625) was passed through a Corning sharp cut filter CS 3-67 (Corning Glass Works, Corning, N. Y.) plus heat filters. The lamp was run at 5 A, 4.6 V, using a regulated power supply. This illumination in 10 min bleached 10% of the rhodopsin of outer segments suspended in a beaker placed at a standard distance (30 cm) from the light source which was used in all experiments. Taking this to reflect a bleaching rate of 1%/min and assuming that each outer segment contains $3 \cdot 10^9$ rhodopsin molecules (17; D. Bownds, unpublished data), this level of rhodopsin bleaching can be expressed (as in the figure legends) as $5 \cdot 10^5$ rhodopsin molecules bleached/outer segment-second. Light was attenuated to obtain lower bleaches (as in the experiments Figs. 1 and 2) with calibrated neutral density filters obtained from Optics Technology, Inc., Palo Alto, Calif.

The magnitude of the volume difference between dark and illuminated outer segments varies from animal to animal. An impression of this variation can be obtained from the statistical treatment shown with Fig. 2. Healthy frogs taken from the field in the fall seem to yield better data than diseased animals, or those taken during the spring and summer mating season.

Outer segments used in the in vitro physiology experiments were prepared for phosphodiesterase assay by sedimentation and resuspension in the modified Ringer's solution mentioned above with papaverine omitted. This procedure caused partial fragmentation of the outer segments. We observed no effects of further fragmentation and lysis. The concentrations of papaverine indicated in Fig. 4 were then added, and phosphodiesterase assay performed according to Kemp and Huang (18, Woodruff et al., in preparation). Cyclic GMP was determined by radioimmunoassay techniques similar to those described by Steiner (19, Woodruff et al., in preparation). Components of the assay were obtained from Collaborative Research, Inc., Waltham, Mass. Other chemicals were obtained from Sigma Chemical Co., St. Louis, Mo., except the phosphodiesterase inhibitors SQ 20-006 and SQ 65-442 (E. R. Squibb & Sons, New Brunswick, N. J.), Ro 20-1724

(Hoffmann-LaRoche, Inc., Nutley, N. J.), and chloropromazine (Smith, Kline & French Laboratories, Inc., Philadelphia, Pa.).

RESULTS

Sensitivity Control System

The swelling of dark rod outer segments can be suppressed by very low levels of illumination (1). To more carefully determine the relationship between illumination and swelling suppression we have performed 39 sensitivity determinations of the sort shown in Fig. 1. In each, the partial suppression of swelling effected by a given level of illumination was determined as a percent of the maximum suppression caused by saturating illumination (which bleached $5 \cdot 10^5$ rhodopsin

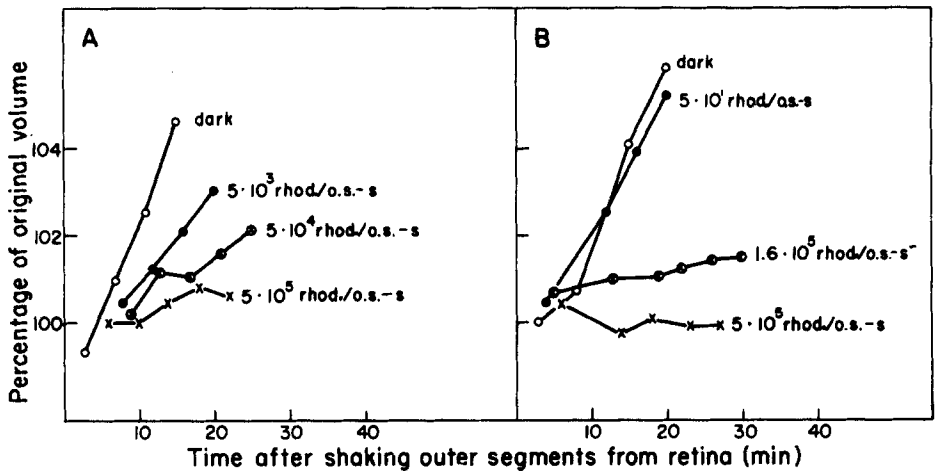

FIGURE 1. Suppression of outer segment swelling caused by different levels of illumination. Different frogs were used in A and B. The curves describe the swelling of separate portions of suspended outer segments, each exposed to the different conditions of illumination which are indicated.

molecules/outer segment-second). The results, collected in Fig. 2, demonstrate that suppression of swelling can be measured over approximately 4 log units of light intensity.

At intensities which bleach between $5 \cdot 10^2$ and $5 \cdot 10^4$ rhodopsin molecules/outer segment-second swelling suppression varies with the logarithm of light intensity. In this range light becomes relatively less effective in suppressing swelling as its intensity is increased. The curve appears similar in shape to the steady-state voltage versus log intensity curve obtained by Baylor and Hodgkin from turtle cones (20) and by Kleinschmidt and Dowling from gecko photoreceptors (21).

Amplitude Control System

We use the word "amplitude" in this in vitro system to refer to the magnitude of the dark swelling or permeability (1) which is suppressible by illumination. Fig. 3

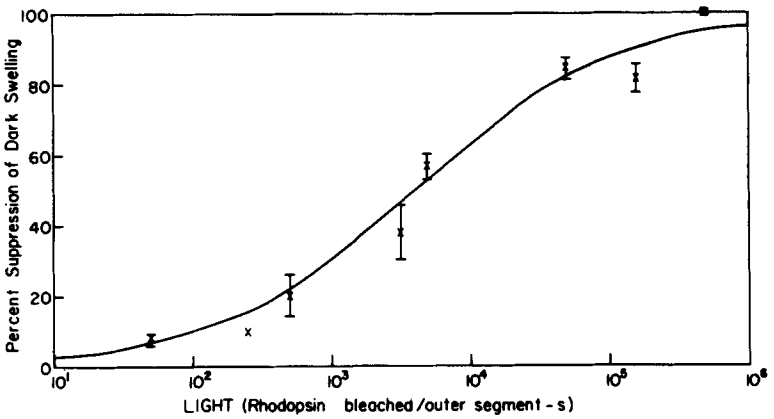

FIGURE 2. Light sensitivity of dark swelling. The points shown were obtained as follows: The best slope (in percent volume increase per minute) of each swelling curve of the sort shown in Fig. 1 was determined by using linear regression analysis. Within each experiment the slope describing rod outer segments maximally bleached with 5×10^5 quanta/outer segment-second was subtracted from all of the other slopes to remove background swelling (1). (Thus this saturating level of illumination is indicated on the figure [■] as causing 100% suppression of swelling.) These corrected slopes describing outer segment swelling in light were divided by the corrected dark swelling slope and this ratio was then subtracted from 1 to yield the percent suppression of swelling caused by each level of illumination. The points represent the mean of the ratios ± sample estimate of the standard error of the mean. All but one of the points represent seven experimental tests. The point without an error bar represents two tests. The solid line was drawn by eye through the data points.

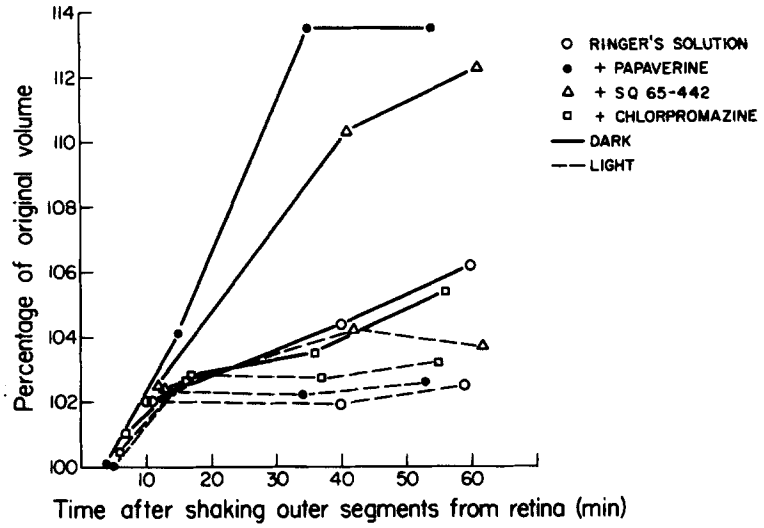

FIGURE 3. Stimulation of light-dependent swelling by phosphodiesterase inhibitors. Solid lines represent swelling of rod outer segments in the dark, dashed lines, those in light which bleached 5×10^5 rhodopsin molecules/outer segment-second. Papaverine (10^{-4} M; ●) and SQ 65-442 (10^{-4} M; △) stimulate dark swelling while chlorpromazine (10^{-5} M; □) has no effect.

illustrates amplitude increases observed after the addition of cyclic nucleotide phosphodiesterase inhibitors. Papaverine addition, for example, increases the dark swelling of outer segments from 0.04–0.1% to 0.13–0.25% of their original volume per minute but has less influence on light-suppressed swelling. Table I summarizes data from many experiments indicating the relative rate at which dark and illuminated outer segments diverge in volume under different conditions in the presence and absence of papaverine. The divergence is most rapid in isosmotic Ringer's solution containing papaverine.

(It is for this reason that we have used this solution, when, as in the sensitivity determinations yielding Fig. 2, large numbers of experiments were required. Under these conditions the ion specificity of the system is perturbed (see Table I); light suppression of swelling can now also be observed with potassium as the major cation present. However, light sensitivity, determined as in Fig. 5 A and B, is not altered. This

TABLE I

Effects of papaverine on light-sensitive swelling of outer segments

	Hyperosmotic Ringer's solution	Isosmotic Ringer's solution
Papaverine added (10^{-4} M)	1.10* (Na^+)	1.96* (Na^+ or K^+)
No addition	0.561* (Na^+)	0.467* (Na^+)

(Light suppression of swelling is observed in the presence of the indicated cations.)

* Relative rate at which dark and illuminated outer segments diverge in volume was analyzed by fitting linear regression relationships to the different conditions, using data from many experiments. (In "hyperosmotic" Ringer's solution, 215 mM NaCl or KCl is used.) Computed slope values were used to determine which conditions most affected swelling. The intercept values were not significantly different from zero at the 99% confidence level, indicating that the outer segments in the light and dark start swelling with equal volumes. By pooling the data in different combinations, the regressions were treated against the null hypothesis that there was no difference between any two conditions. The "F" test values were much greater than the critical F value at the 99% confidence level (25). This demonstrates that all the conditions influenced the outer segment's reaction to light in different ways.

apparent relaxing of ion specificity does not extend to other ions, for rapid dark swelling is still not seen in LiCl. Further, an inhibition of swelling caused by Ca^{++} ions which we noted earlier [1] has the same concentration dependence with or without added papaverine.)

Stimulation of swelling is observed not only with papaverine but also with other phosphodiesterase inhibitors such as SQ 65-442 (Fig. 3), SQ 20-006, isobutyl methylxanthine, and Ro 20-1724 (latter three not illustrated). Because hydrophobic amines such as papaverine, chlorpromazine, and colchicine can cause membrane conformation changes not related to phosphodiesterase inhibition (22), we examined the effects of the latter two drugs. Neither chlorpromazine (10^{-5} to 5×10^{-4} M, Fig. 3) nor colchicine (10^{-4} M, not illustrated) mimicked papaverine. Further evidence that papaverine acts by inhibiting cyclic nucleotide phosphodiesterase in rod outer segments comes from the dose-response curve shown in Fig. 4. Concentrations of papaverine (10^{-4} M) maximally effective in inhibiting cyclic nucleotide phosphodiesterase activity are also most effective in stimulating dark swelling. (At higher concentrations papaverine decreases the

volume difference between dark and illuminated outer segments and at 1–10 mM levels causes their fragmentation.)

Finally we have directly measured cyclic GMP levels in these outer segment preparations and have found that papaverine inhibition of phosphodiesterase is accompanied by an increase in cyclic GMP concentration. After outer segments break from the retina, cyclic GMP levels decay within 10 min to a relatively steady value of approximately 0.01 mol cyclic GMP/mol rhodopsin present. If 10^{-4} M papaverine is present twice as much cyclic GMP remains. In either case,

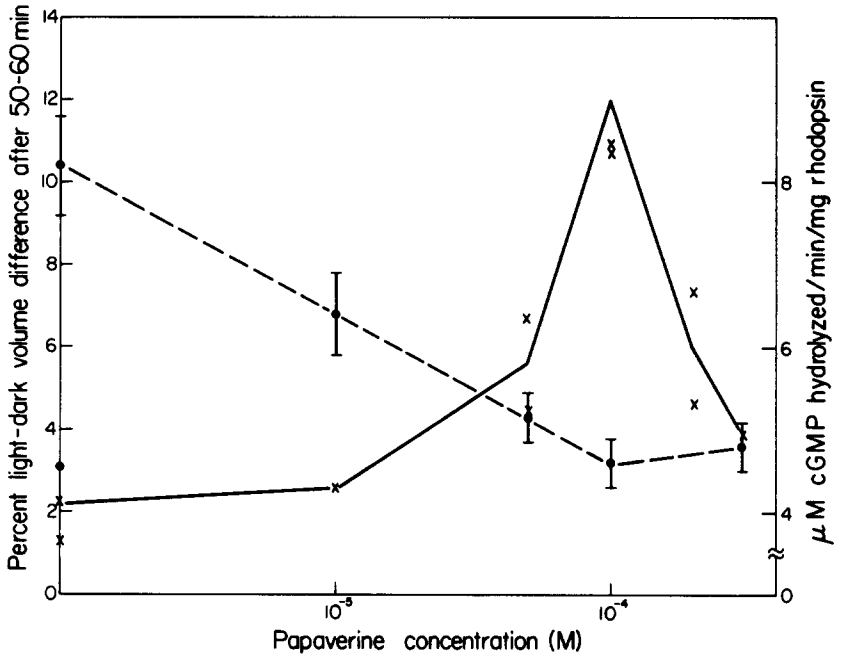

FIGURE 4. Papaverine dose-response curve. Stimulation of dark swelling by papaverine is indicated as the percent difference in volume between illuminated and dark outer segments after 50–60 min in Ringer's solution containing the concentrations of papaverine indicated (solid line, left ordinate). Inhibition of phosphodiesterase activity (dashed line, right ordinate) is indicated for the same papaverine concentrations.

illumination which activates the phosphodiesterase lowers cyclic GMP levels by at least a factor of 2. (We have not yet carefully measured the effect of papaverine on cyclic AMP levels because it is present in 100-fold lower concentration than cyclic GMP and is more difficult to assay. Measurements directly relating cyclic GMP changes to in vitro physiology are presented in more detail in a paper to follow.)

Pharmacological Separation of Sensitivity and Amplitude Control

Papaverine has a pronounced effect on dark swelling, or amplitude, but it does not appear to greatly alter the light sensitivity of the in vitro system. Fig. 5 A and

B indicate that the relative suppression of swelling effected by differing levels of illumination remains approximately the same in the presence and absence of papaverine. (This is a qualitative statement, for we have not done the number of experiments required to present the data as in Fig. 2.) In addition, in vitro "dark adaptation" (1) remains the same with papaverine (data not shown). Papaverine is serving here as a pharmacological probe permitting us to distinguish the sensitivity-controlling system, which it does not affect, from the amplitude-controlling system, which it does affect. Consistent with this distinction there appears to be no effect of papaverine on the rhodopsin phosphorylation reac-

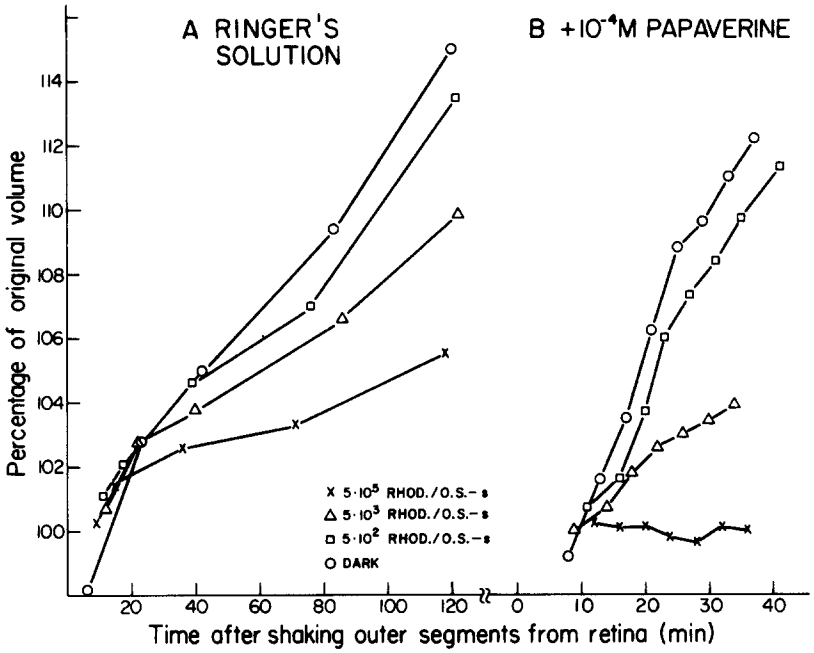

FIGURE 5. Light sensitivity in isosmotic Ringer's solution is relatively unaffected by papaverine addition. Rod outer segments, after dilution, are put into the dark (○); or continuous light bleaching 5×10^2 rhodopsin molecules/outer segment-second (□); 5×10^3 rhodopsin molecules/outer segment-second (△); or 5×10^5 rhodopsin molecules/outer segment-second (×).

tion (see reference 23). (Conversely, phosphorylation inhibitors increase sensitivity but do not influence the maximum change in swelling which can be caused by illumination [2].)

DISCUSSION

The sensitivity measurements shown in Fig. 2 extend our previous work (1) to demonstrate that isolated outer segments can perform not only dark adaptation, but also a form of sensitivity control. Their responsiveness to light decreases as light intensity increases. Moreover, we can observe suppression of swelling or permeability as light intensity increases over 3–4 log units. This raises the

possibility that the response may be the in vitro analog of the desensitized, or "steady-state," response of living receptor cells to continuous illumination (20, 21). Further, the distinction made between sensitivity and amplitude control in this in vitro system appears to parallel the separation of sensitivity and membrane voltage observed in living gecko photoreceptors (21).

Having developed procedures for measuring sensitivity, we have begun experiments to determine whether it can be altered by various chemical perturbations. We have found that outer segments incubated with adenosine or β,γ-methylene ATP, both inhibitors of light-activated rhodopsin phosphorylation, become more sensitive to illumination (2). For example, bleaching 5×10^3 rhodopsin molecules/outer segment-second, which normally causes half-maximal suppression of swelling in untreated outer segments, causes 75% of maximal suppression of swelling if 1–10 mM adenosine is present. From Fig. 2 we can estimate that this additional suppression of swelling would, in the absence of the phosphorylation inhibitor, be caused by a 10-fold increase in illumination. A second inhibitor of rhodopsin phosphorylation, β,γ-methylene ATP, is even more effective than adenosine in increasing sensitivity. Maximum suppression of dark swelling, normally caused by light bleaching 5×10^5 rhodopsin molecules/outer segment-second, is effected by 100-fold times less illumination in the presence of this compound (2).

The data give no information on mechanisms by which phosphorylation of rhodopsin might decrease sensitivity. It is possible that phosphorylation inactivates the ability of rhodopsin to perform in transduction. Alternatively, phosphorylation might activate an enzymic activity of rhodopsin which regulates the concentration of the internal transmitter which is commonly presumed to travel between the disk and plasma membranes after illumination (24). Many other possibilities, including that the correlation we observe is fortuitous, cannot be excluded.

The observed correlation between phosphodiesterase inhibition and increases in maximum amplitude can also be interpreted in several different ways. Aside from being a phosphodiesterase inhibitor, papaverine might have other influences in the system. This possibility is raised by the observation that papaverine perturbs ion specificity of the in vitro assay under some of the conditions used. We do not know whether this perturbation is a consequence of changing cyclic GMP levels.

While the pharmacological data, along with the direct measurements of cyclic GMP levels, do suggest an involvement of cyclic GMP in outer segment physiology, the actual mechanisms will be established only when the relevant cyclic nucleotide-related chemistry has been directly measured. Cyclic GMP, for example, might influence the number of permeability sites of the plasma membrane available for interaction with an internal transmitter, influence mobilization of the transmitter or its binding to permeability sites, regulate some aspect of the "vegetative" metabolism of outer segments required for maintaining open permeability sites, or be one of the internal transmitters which directly regulates permeability.

While it would be premature to offer a detailed model in the face of all these possible mechanisms, we favor the general suggestion that the disk membrane

system, where most rhodopsin phosphorylation occurs, is the primary site of sensitivity control. Amplitude control, possibly effected by cGMP, might be centered on the plasma membrane which contains the regulated permeability mechanism. The experiments of this paper, together with our earlier work (2), provide a clear pharmacological separation of these two controlling systems, a separation which will be useful in further biochemical studies.

We are grateful to Mr. Michael Woodruff and Mr. Steven Green for permitting us to cite some of their data before its publication.

This work was supported by NIH grant EY-00463.

Received for publication 5 January 1976.

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