Properties of the pH-sensitive Site that Controls the λ_{max} of *Limulus* Metarhodopsin

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ABSTRACT A pH-sensitive site controls the λ_{max} of *Limulus* metarhodopsin. The properties of this site were examined using intracellular recordings of the early receptor potential (ERP) as a pigment assay. ERPs recorded over a range of extracellular pHs indicate that the apparent pK of the site is in the range of 8.3–8.6. Several lines of evidence indicate that the site responds directly to changes in extracellular pH (pH_o) rather than to changes in intracellular pH (pH_i) that follow as a secondary result of changing pH_o: (*a*) the effect of changing pH_o was rapid (<60 s); (*b*) when pH_o was raised, the simultaneous rise in pH_i, as measured with phenol red, was relatively small; (*c*) raising pH_i by intracellular injection of pH 10 glycine buffer did not affect the site; and (*d*) the effect of changing pH_o could not be blocked by increasing the intracellular pH buffering capacity. It is concluded that the pH-sensitive site on metarhodopsin is on the extracellular surface of the plasma membrane.

INTRODUCTION

Physiological techniques have been useful in identifying binding sites on membrane proteins that contribute to the electrical properties of nerve membrane. In many cases it has been possible to determine whether these sites are on the intracellular or the extracellular surface of the plasma membrane. For instance, in squid axon the receptor for tetrodotoxin on the sodium channel is accessible only from the extracellular space (Narahashi et al., 1966), whereas the receptor for tetraethylammonium chloride on the potassium channel is accessible only from the intracellular space (Armstrong and Binstock, 1965).

Rhodopsin is a membrane glycoprotein that can be solubilized and studied biochemically. Binding sites on various solubilized visual pigments have been identified for the following substances: hydroxylamine, sulfhydryl reagents, protons, chloride ions, retinal, proteolytic enzymes, and agents that bind to sugars. In the case of vertebrate rhodopsin, the location of some of these sites has been identified with a variety of chemical and cytological techniques (for a review see Hubbell and Fung, [1979]). In this report we describe physiological experiments on intact *Limulus* photoreceptors that demonstrate that an important proton binding site on metarhodopsin is on the extracellular surface of the plasma membrane.

J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/81/02/0191/13 $1.00 191
Volume 77 February 1981 191–203

Metarhodopsin is a photoproduct of rhodopsin and, in invertebrates, is thermally stable. Hubbard and St. George (1958) showed that squid (*Loligo*) metarhodopsin is a pH indicator: raising the pH converts acid metarhodopsin (λ_{max} 500 nm) to alkaline metarhodopsin (λ_{max} 380 nm). They further showed that the pH sensitivity is due to a single site with a pK of 7.7. The site has also been demonstrated in living squid photoreceptors using the early receptor potential (ERP) (Hagins and McGaughy, 1967). When either form of metarhodopsin absorbs a photon, metarhodopsin is converted to rhodopsin or isorhodopsin.

Limulus ventral photoreceptors contain a visual pigment similar to that of the squid (Lisman and Sheline, 1976). Because of the large size of the ventral photoreceptors, it has been possible to manipulate extracellular and intracellular pH and thereby to determine the orientation of the pH-sensitive site on metarhodopsin. A preliminary report of our findings has been published (Lisman et al., 1975).

MATERIALS AND METHODS

Microelectrodes filled with 3 M KCl and having resistances of 10–20 MΩ were used for intracellular recording of the ERP. Electrodes with larger tips were used in experiments in which pressure injection was required. In these experiments the same electrode was used for pressure injection and for recording the ERP. To facilitate electrode impalement, the preparation was treated with pronase (2 mg/ml) for 1 min. The dissection and impalement were performed under lights sufficiently bright to produce a photoequilibrium of rhodopsin and metarhodopsin (see Fig 1 *a*).

ERPs were evoked by flashes from a Strobonar 710 (Honeywell, Inc., Test Instruments Div., Denver, Colo.). Light from this source was filtered to transmit wavelengths longer than 455 nm (GG 455 filter; Schott Optical Glass Inc., Duryea, Pa). The exhaustive bleaches at high pH were 45-s exposures to 430-nm light (3×10^{-4} W/cm^2). The optical stimulator was as described in Lisman and Strong (1979).

For determining the pK of metarhodopsin, cells were superfused with a series of seawaters with different pHs. Solutions having a pH of 10.2 and 9.5 were buffered with 30 mM glycine and, to avoid the formation of MgOH precipitate, contained only 1 mM Mg. Solutions having a lower pH were buffered with 30 mM tris(hydroxymethyl)aminomethane (Tris). pH was measured before and after each experiment. For the purpose of constructing a titration curve, the pH was taken as the mean of these two values. The change in pH during the time required for the experiment (5–6 h) was usually <0.1 pH unit. The perfusion chamber had a volume of 0.05 ml, and the flow rate was 1 ml/min.

RESULTS

Fig. 1 illustrates the pH sensitivity of *Limulus* metarhodopsin as assayed by the ERP. All the ERPs described in this paper were recorded with an intracellular microelectrode and were evoked by brief flashes filtered to transmit wavelengths longer than 455 nm. Under these conditions, rhodopsin and acid metarhodopsin contributed to the ERP, but alkaline metarhodopsin did not, because it did not absorb the wavelengths of the flash. Absorption of light by acid metarhodopsin generated a monophasic positive wave, whereas absorption of light by rhodopsin generated a more slowly rising monophasic negative wave (Lisman and Sheline, 1976).

Fig. 1 *a* shows the biphasic ERP at pH 7.36 generated by a photoequilibrium mixture of rhodopsin and acid metarhodopsin. When the extracellular pH was raised to 10.2, most of the acid metarhodopsin was converted to alkaline metarhodopsin. Under these conditions, a flash evoked a large monophasic negative response generated almost solely by rhodopsin (Fig. 1 *b*). The effect of high pH on the ERP was almost complete within 1 min; however, to ensure a maximum effect, cells were exposed to high pH for 3 min before ERPs were evoked. After exhaustive 530-nm irradiation, there was little rhodopsin or acid metarhodopsin remaining, and a flash evoked only a small residual ERP (Fig.

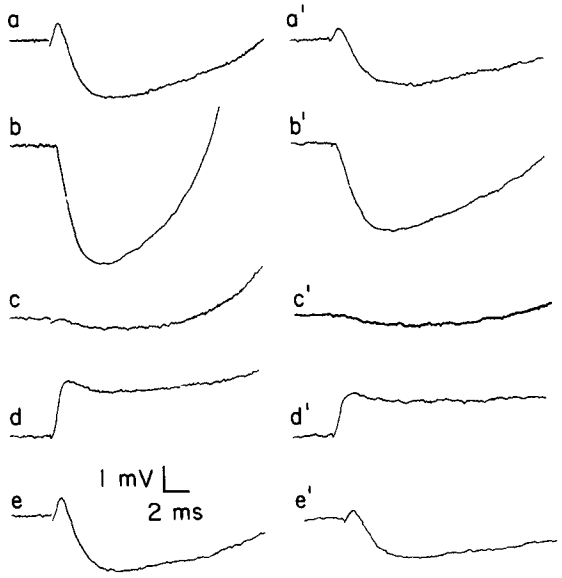

FIGURE 1. Effects of high pH and irradiation on the ERP. (*a*) Biphasic ERP measured under photoequilibrium conditions at pH 7.36. (*b*) Monophasic negative ERP in response to first flash after pH was raised to 10.2. (*c*) Residual ERP at pH 10.2 after exhaustive 530-nm irradiation. (*d*) Monophasic positive ERP in response to first flash after pH was lowered to 7.36. (*e*) Biphasic ERP after several flashes at pH 7.36. *a'–e'* are the same as above except recorded later in the experiment after seven intervening exposures to pH-10.2 solution. The very rapid negative-going deflection at the beginning of some responses (for example, *a*, *c*, and *e*) is a brief (500 μs) electrical artifact generated by the onset of the strobe flash.

1 *c*). Most of the pigment was then alkaline metarhodopsin that could be converted to acid metarhodopsin by lowering the pH to 7.36 in the dark. Under these conditions, the ERP was a large monophasic positive response (Fig. 1 *d*). After exhaustive illumination a photoequilibrium mixture of rhodopsin and acid metarhodopsin was reestablished, and the ERP was again biphasic (Fig. 1 *e*). This cycle could be repeated indefinitely; however, repeated exposure of the preparation to high pH led to a gradual reduction in ERP amplitudes. For example, after the procedure used to generate Fig. 1 *a–e* was repeated eight times, the ERPs were smaller, as shown in Fig. 1 *a'–e'*.

The pK of Metarhodopsin

To measure the titration of metarhodopsin, we followed a protocol similar to that described above, except that instead of returning from pH 10.2 to 7.36, pH was lowered to some intermediate value [pH(x)], and the ERP measured. At each intermediate pH the ERP was a monophasic positive wave smaller than the monophasic positive ERP at pH 7.36 (Fig. 2; *inset,* Fig. 3 *a*), because alkaline metarhodopsin was only partially converted to acid metarhodopsin. To compare quantitatively the ERP at pH(x) to that at pH 7.36, it was

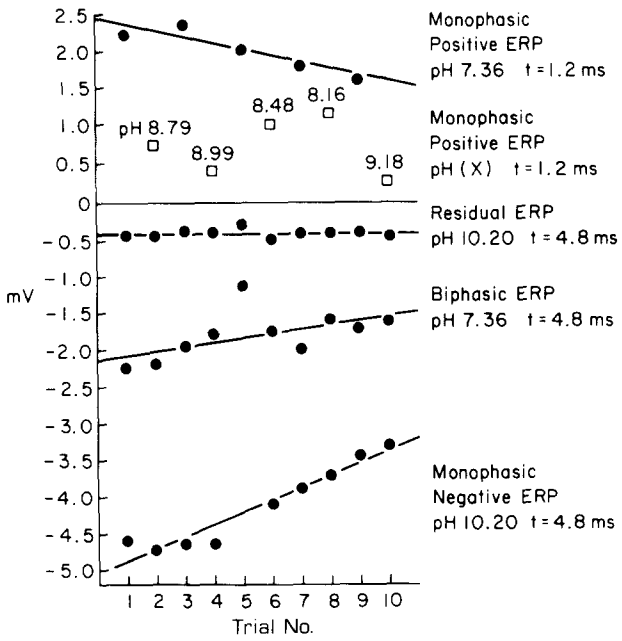

FIGURE 2. ERP amplitudes during successive trials of the kind described in Fig. 1 *a–e*. After pH had been raised to 10.2 and irradiation as described in the text had been carried out, the pH was returned either to 7.36 or to some intermediate pH(x). The solid lines were computed by linear regression. Voltages were measured at 1.2 and 4.8 ms after the onset of the flash, because these were, respectively, the peak of the large monophasic positive responses and the peak of negative component of the large biphasic responses.

necessary to use a procedure that took into consideration the reduction of ERP amplitude that occurred with each successive exposure of the cell to high-pH seawater. This was accomplished by alternating trials at pH(x) with trials at pH 7.36, computing the linear regression line that describes the monophasic positive response at pH 7.36 as a function of trial number (uppermost line in Fig. 2), and then comparing the response at pH(x) to the response at the same trial number predicted by the regression line for responses at pH 7.36. The ratio of the response at pH(x) to the response at pH 7.36 is plotted in Fig. 3 *a*.

To determine the titration curve of metarhodopsin, a measure of the

amount of acid metarhodopsin at different pHs is needed. The monophasic positive ERPs shown in the *inset* of Fig. 3 *a* may be used to estimate the metarhodopsin concentration, provided the small contribution of rhodopsin is corrected for. The reason for suspecting a contribution of rhodopsin is that the residual ERP at pH 10.2 has a negative component. The rhodopsin that

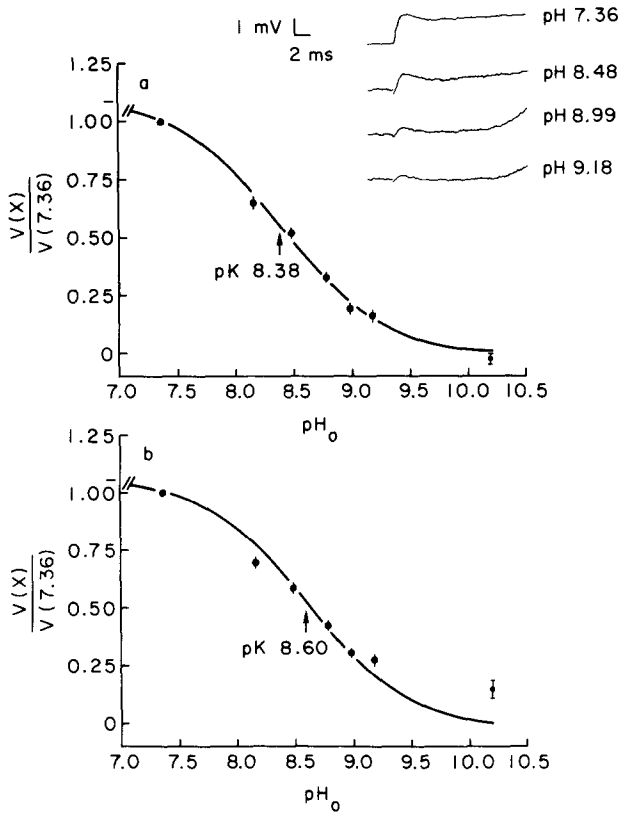

FIGURE 3. Titration curves for the pH-sensitive site on metarhodopsin. (*a*) Points are the ratios of monophasic positive response at pH(x) to monophasic positive response at pH 7.36. Some typical responses are shown in the *inset*. The solid curve is the titration curve for a single site with pK 8.38. The point at pH 10.2 was measured during each trial, and the mean and standard deviation are shown. (*b*) The data in *a* has been replotted after correction for contribution of rhodopsin to the monophasic positive responses according to assumption *a* described in the Appendix. The solid line is the titration curve for a site with pK 8.60. The marks on the ordinate between 1 and 1.25 signify the V_{max} of the two titration curves.

generates this negative wave must exist in photoequilibrium with a fraction of acid metarhodopsin that has not been converted to the alkaline form. Lowering the pH from 10.2 would not be expected to eliminate the negative rhodopsin wave, and it would, therefore, reduce the amplitude of all monophasic positive ERPs.

The required correction depends on the reason for the residual rhodopsin and acid metarhodopsin. Two possible reasons and the related corrections are described in detail in the Appendix. Briefly, if it is assumed that all metarhodopsin is exposed to the same extracellular pH, then the data in Fig. 3 *a* must be corrected as shown in Fig. 3 *b*. These corrected points can be fit moderately well between pH 7.36 and 8.99 by a titration curve for a single site with a pK of 8.60 (8.59 and 8.57 in two other cells). However, because of the large size of the residual ERP, the data points at pH 9.18 and 10.2 are much larger than expected on the basis of a single site with this pK. Alternatively, it may be assumed that a small fraction of acid metarhodopsin cannot be converted to alkaline metarhodopsin, because it is relatively inaccessible to the superfusate or because it is inherently insensitive to pH. This fraction would give rise to the residual ERP. In this case, the needed correction is negligible (see Appendix) and the data in Fig. 3 *a* can be considered a valid quantitative measure of the fraction of metarhodopsin that can be affected by external pH. These data are nicely fit over the entire pH range by the titration curve for a single site with a pK of 8.38 (8.45 and 8.52 in two other experiments).

Although the large size of the residual ERP at pH 10.2 is probably due to a fraction of metarhodopsin that is not affected by changing the pH of the bath, it is possible to imagine another explanation. The ERP amplitude depends not only on the number of pigment molecules isomerized by the flash, but also on the magnitude of the light-induced dipole shift within the visual pigment molecules. It is possible that the large size of the residual ERP is due to a small number of molecules that generate a large ERP because high pH has increased the dipole shift. Indeed, there is evidence that high pH has this effect (Fig. 5 of Lisman and Sheline, 1976). Nevertheless, the residual ERP cannot be accounted for in this way, because this model erroneously predicts that bleaching pigment at high pH should lead to a very large reduction in pigment concentration and consequently to a very large reduction in the sensitivity of the late receptor potential. However, Lisman and Strong (1979) found that the changes in the sensitivity of the late receptor potential were less than tenfold and that these changes could be adequately predicted using the amplitudes of the early receptor potential.

Localization of the pH-sensitive Site

That the λ_{max} of metarhodopsin is affected by changing extracellular pH (pH_o) does not prove that the site is on the extracellular surface. The site might be on the inside surface and respond to changes in intracellular pH (pH_i) that occur secondarily as a consequence of changes in pH_o. As mentioned, raising pH_o produces an effect on metarhodopsin that is almost complete within 1 min. Lisman and Strong (1979) measured pH_i after raising pH_o from 7.8 to 10, and from their data (their Fig. 12) it can be estimated that during the first minute pH_i rose by only ~0.15 pH unit. Using the Harvard microspectrophotometer (Brown, 1961) and the phenol red calibration curves in Lisman and Strong (1979), we have carried out similar experiments and found that, even during much longer periods in high-pH (9.4) seawater, pH_i rises by <0.5 units. Thus, pH_i is relatively insensitive to pH_o.

Although the changes in pH_i caused by raising pH_o are small, they might still be sufficient to affect a pH-sensitive site on the internal surface of the plasma membrane. To examine this possibility we raised pH_i without changing pH_o. Fig. 4 shows how intracellular injection of pH-10 glycine buffer affected the ERP. Before the injection, the ERP had a biphasic wave form typical of photoequilibrium conditions at pH 7.8. The rapidly depolarizing component at the far right in Fig. 4 *a* is the rising edge of the late receptor potential. Fig. 4 *b* shows the effect of an initial injection of pH-10 buffer. The positive component of the ERP was hardly affected, but the amplitude of the

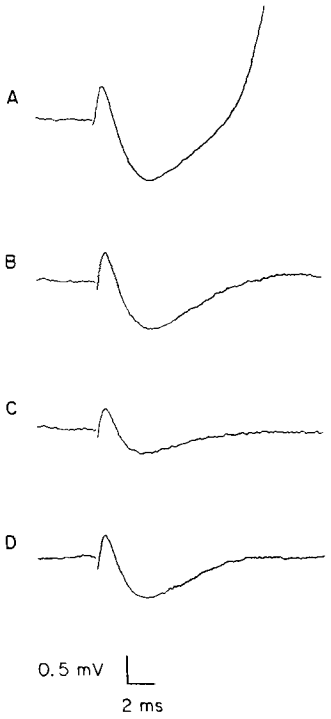

FIGURE 4. Effects of intracellular injection of pH-10 glycine buffer on ERP. (*a*) Biphasic ERP recorded before injection. (*b*) ERP recorded toward the end of initial injection. (*c*) ERP recorded toward end of second injection. (*d*) ERP recorded several minutes after end of second injection.

negative component was reduced. Resting potential was reduced by 20 mV (not shown), and the late receptor potential was completely abolished. The negative component of the ERP was further reduced by a second injection (Fig. 4 *c*) from which there was a partial recovery (Fig. 4 *d*). The recovery demonstrates that the reduction in ERP amplitude was not due merely to change in the quality of the electrode impalement. The principal conclusion to be drawn from Fig. 4 is that the effects of raising pH_i differ entirely from the effects of raising pH_o: there is no indication of a conversion of acid to alkaline metarhodopsin; such a conversion would have produced a large increase in the negative component of the ERP. It follows that the pH-

sensitive site on metarhodopsin that controls its λ_{max} must be on the extracellular surface.

An objection that might be raised to this argument is that pH_i was not measured during the experiments in Fig. 4, and so there is no proof that pH_i was actually raised by the injection of pH-10 buffer. Several lines of argument suggest that pH_i was in fact substantially raised. First, in one experiment we injected both phenol red and pH-10 buffer and measured the absorbance spectrum in the microspectrophotometer. These measurements showed that pH_i was higher than 7.8, the upper limit of resolution of our method. Second, on the basis of experiments in other cells, the injection of pH-10 buffer should have been sufficient to overcome the endogenous buffer. Because the ventral photoreceptors were observed to swell after injection, the injection must have been at least 20% of a cell's volume. The 0.5 M glycine injection solution would therefore be diluted to 100 mM in the cytoplasm. This would produce a buffer capacity of about 50 slykes, much larger than the 15-slyke value measured in barnacle photoreceptors (Brown and Meech, 1979). Finally, Coles and Brown (1976) injected a variety of pH buffers into the cytoplasm of *Limulus* ventral photoreceptors and found that varying the pH of injection solution from 5.4 to 8.4 had a systematic effect on the kinetics of light-induced current. However, in no case did they find any dramatic effect on the amplitude of the receptor potential. In contrast, injection of pH-10 glycine buffer (Fig. 4) completely abolished the late receptor potential. These findings make it extremely likely that injection of pH-10 buffer actually caused a large elevation of pH_i.

Another way of determining whether changes in pH_o affect an intracellular site via a change in pH_i is to stabilize pH_i with buffer. To do this we injected the pH buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid) (HEPES) (pH 7.0; 0.5 M), into the cytoplasm until the cell appeared swollen. After such injections the kinetics of the late receptor potential were greatly slowed, which is indicative of injections that are a significant fraction of a cell's volume (Lisman and Brown, 1975). The added buffer concentration would be 100 mM if it is assumed that the injection volume was 20% of the cell volume. After the injection, the ERP was normal. pH_o was then raised to 9.6. After 2 min, the ERP was a large monophasic negative response, similar to that in cells not injected with buffer. Thus, increasing the intracellular buffering capacity did not block the conversion of acid metarhodopsin to alkaline metarhopsin, further supporting the conclusion that the pH-sensitive site on metarhodopsin is on the extracellular surface of the plasma membrane.

DISCUSSION

The λ_{max} of *Limulus* metarhodopsin is controlled by a pH-sensitive site. We have found that the effective pK of the site is between 8.3 and 8.6 (Fig. 3). This is the first determination of the pK of metarhodopsin *in situ;* however, the pK of extracted metarhodopsin has been measured in several Cephalopods, and varies from 9.1 in the squid *Todarodes pacificus* to 7.3 in *Octopus orellatus* (Hara and Hara, 1972). In making the comparison between measurements on

solubilized proteins and proteins *in situ,* it is important to keep in mind that the pH near a membrane protein may differ from the pH of the bulk solution, because the negative surface potential and the membrane potential itself can concentrate protons near the membrane and, thereby, raise the apparent pK. Both these mechanisms affect the apparent pK of a pH-sensitive site in the sodium channel of the node of Ranvier (Woodhull, 1973). If the surface potential in *Limulus* is −46 mV, as in squid axon (Gilbert, 1971), and if there is no effect of membrane potential, the true pK can be calculated, as in Woodhull (1973), to be 7.5–7.8.

All the experimental evidence indicates that the pH-sensitive site that controls the λ_{max} of metarhodopsin is on the extracellular surface of the plasma membrane: the effect of raising pH_o on metarhodopsin was rapid (<1 min). During this period the change in pH_i was considerably less than 0.2 pH units. Separately raising pH_i did not convert acid metarhodopsin to alkaline metarhodopsin. Furthermore, the effect of raising pH_o could not be blocked by increasing the pH buffering capacity of the cytoplasm. It is thus highly unlikely that the site is on the inside surface of the plasma membrane and that it responds to changes in pH_i that follow secondarily from changes in pH_o.

The only other information about the location of invertebrate rhodopsin in the membrane comes from freeze-fracture studies. In invertebrates (Fernandez and Nickel, 1976), including *Limulus,*[1] membrane particles are found primarily in the cytoplasmic leaflet of the plasma membrane. This finding suggests that rhodopsin is exposed to the intracellular space but in no way rules out exposure to the extracellular space. The techniques used to show that vertebrate rhodopsin is a transmembrane protein (Hubbel, 1977) have not been applied to invertebrate visual pigments.

Implications for Visual Physiology

In the living animal, pH_o is unlikely to change sufficiently to alter the acid metarhodopsin concentration. It is thus unlikely that the pH-sensitive site on metarhodopsin is a physiologically significant control mechanism. The simplest assumption about the nature of the site is that it is the Schiff's base linkage between the chromophore and protein and that it is exposed in metarhodopsin but not in rhodopsin (Hubbard and St. George, 1958). This inference is based on the finding that conjugates of retinal and amino compounds show pH indicator properties similar to that of metarhodopsin (Ball et al., 1949).

The external location of the pH-sensitive site is important experimentally because of the ease with which pH_o can be changed. By raising pH_o and irradiating with long-wavelength light, Lisman and Strong (1979) were able to lower the rhodopsin concentration in the living photoreceptor and to study the resulting effects on excitation and adaptation. A roughly tenfold concentration change appears to be the maximum that can be achieved by this

[1] Wong, F., and H. B. Peng. Personal communication.

method. As shown in Fig. 1 *c* and *e*, there is a small ERP evoked by long-wavelength light that we have been unable to eliminate by further irradiation or longer exposure to high pH. Although the interpretation of this finding is uncertain, a likely explanation is that a small fraction of the microvillar surface is not accessible to the superfusate.

Perhaps more importantly, our experiments show that at least 80% of the microvillar surface is rapidly affected by changing pH_o. The ERP generated by metarhodopsin provides a measure of the average pH on the extracellular surface of the villi. Inasmuch as a large fraction of the metarhodopsin can be described by a titration curve for a single site (Fig. 3), most of the intervillar space must be rapidly penetrated by superfusate. This is consistent with previous cytological studies demonstrating that lanthanum, added extracellularly, penetrates into the region between the microvilli (Perrelet and Baumann, 1969).

In the discussion of Fig. 1 and similar experiments in previous papers (Lisman and Sheline, 1976; Lisman and Strong, 1979), we assumed that at physiological pH the ventral photoreceptor contained only rhodopsin and acid metarhodopsin. We now believe this interpretation should be slightly modified. If the pK of metarhodopsin is 8.4, then ~10% of the pigment should be alkaline metarhodopsin at physiological pH (7.8). Furthermore, if it is assumed that, as in squid, absorption of light by alkaline metarhodopsin can produce isorhodopsin (Hubbard and St. George, 1958), then the ventral photoreceptor may also contain isorhodopsin. The physiological effects of light absorbed by isorhodopsin or alkaline metarhodospin are unknown.

APPENDIX

After long-wavelength irradiation at pH 10.2, there is a residual biphasic ERP (Fig. 1 *c* and *c'*). We assume that the negative component of this residual ERP is generated by rhodopsin that exists in a photoequilibrium with a small population of acid metarhodopsin. The existence of this residual rhodopsin implies that the monophasic positive ERPs at various pHs (Fig. 1 *d* and *d'* and *inset*, Fig. 3 *a*) cannot be attributed solely to acid metarhodopsin and that a correction must be made for the contribution of rhodopsin to these responses. We have considered two reasons for the residual acid metarhodopsin, which yield quite different corrections: (*a*) All metarhodopsin is equally pH sensitive and is exposed to the same extracellular pH at any given time. (*b*) A small fraction of metarhodopsin cannot be converted from acid metarhodopsin to alkaline metarhodopsin by our procedures, either because the molecules are inherently pH insensitive or because they are inaccessible to pH-10.2 perfusate. A set of definitions is described below. The corrections that follow from assumptions *a* and *b* are then derived.

$V_b(t)$	wave form of the biphasic ERP at pH 7.4 (e.g., Fig. 1 *a*)
$V_-(t)$	wave form of the first monophasic negative ERP at pH 10.2 (e.g., Fig. 1 *b*)
$V_r(t)$	wave form of the residual ERP at pH 10.2 (e.g., Fig. 1 *c*)
$V_+(t)$	wave form of the first monophasic positive response at pH(x)(e.g., Fig. 1 *d* and *inset* Fig. 3 *a*)

Assume that each of these is the linear sum of contributions from the rhodopsin [$R(t)$] and acid metarhodopsin [$M(t)$] present under each condition.

$$V_b(t) = M_b(t) + R_b(t) \tag{1}$$

$$V_-(t) = M_-(t) + R_-(t) \tag{2}$$

$$V_r(t) = M_r(t) + R_r(t) \tag{3}$$

$$V_+(t) = M_+(t) + R_+(t) \tag{4}$$

Each term on the right-hand side of Eqs. 1–4 depends not only on the number of pigment molecules in a given state but also on the light-induced dipole shift within each molecule. The dipole shift may itself be pH dependent (Lisman and Sheline, 1976). We have not corrected for this possibility, because we have no independent measure of it and because, in any case, the influence on the titration curve would be small.

Corrections Based on Assumption a

Using Eq. 4, $M_+(t)$ can be computed from $V_+(t)$ if $R_+(t)$ is known. $R_+(t)$ should be the same as the rhodopsin contribution [$(R_r(t)$] to the residual responses, because the rhodopsin concentration is not changed by lowering the pH in the dark. Therefore,

$$M_+(t_1) = V_+(t_1) - R_+(t_1) = V_+(t_1) - R_r(t_1). \tag{5}$$

Let t_2 be the time at which the biphasic ERP [$V_b(t)$] has its negative peak. Consider the quantity

$$B = \frac{R_b(t_1)}{V_b(t_2)}. \tag{6}$$

From 6 and 1

$$B = \frac{R_b(t_1)}{R_b(t_2) + M_b(t_2)} = \frac{1}{\dfrac{R_b(t_2)}{R_b(t_1)} + \dfrac{M_b(t_2)}{R_b(t_1)}}. \tag{7}$$

Because the rightmost expression in Eq. 7 depends only on the ratios of responses, B does not depend on the amount of pigment in the photoequilibrium. Because changing pH changes only the amount of rhodopsin and acid metarhodopsin in photoequilibrium, B, which in Eq. 7 applies to pH 7.4, must have the same value at pH 10.2. Thus,

$$B = \frac{R_b(t_1)}{V_b(t_2)} = \frac{R_r(t_1)}{V_r(t_2)}. \tag{8}$$

Rearranging terms,

$$R_r(t_1) = \frac{R_b(t_1)}{V_b(t_2)} \cdot V_r(t_2). \tag{9}$$

To estimate $R_b(t_1)$, consider the large monophasic negative response $V_-(t)$ that occurs after pH_0 is raised to 10.2. This response is generated almost solely by rhodopsin, because most of the acid metarhodopsin that contributed to the large biphasic response at pH 7.4 has been converted to alkaline form. Therefore, $M_-(t) = 0$, and from Eq. 2, to a good approximation,

$$V_-(t_1) = R_-(t_1). \tag{10}$$

Since rhodopsin concentration is not changed by pH,

$$R_{-}(t_1) = R_b(t_1). \tag{11}$$

Combining Eqs. 5, 9, 10, and 11 yields

$$M_{+}(t_1) = V_{+}(t_1) - \frac{V_{-}(t_1)}{V_b(t_2)} \cdot V_r(t_2).$$

For each trial at a given pH, values of $V_{+}(t_1)$, $V_b(t_2)$, and $V_r(t_2)$ were determined for the same trial number from linear regression curves (e.g., Fig. 2), and $M_{+}(t_1)$ was computed. Corrected ratios for $M_{+}[\text{pH}(x)]/M_{+}(\text{pH } 7.36)$ are plotted in Fig. 3 *b*. Between pH 7.36 and 9.2, a titration curve for a site with pK 8.6 fits the data moderately well, but at pH 10.2 the data point is much higher than predicted. This might mean that the λ_{max} of metarhodopsin is controlled by more than one pH-sensitive site; however, a viable single-site model based on assumption *b* is described below.

Correction Based on Assumption b

We assume that at pH 10.2 the residual ERP is generated by a fraction of metarhodopsin not influenced by raising the pH to 10.2. A small amount of rhodopsin exists in photoequilibrium with this metarhodopsin. Thus,

$$V_r(t) = M_r^{-}(t) + R_r^{-}(t), \tag{12}$$

where the minus sign designates the subset of visual pigment not affected by pH. If $M_{+}^{*}(t)$ designates the response generated by the subset of acid metarhodopsin which *can* be affected by high pH, then

$$V_{+}(t) = M_{+}^{*}(t) + M_{+}^{-}(t) + R_{+}^{-}(t). \tag{13}$$

Since the terms designated by a minus sign are by definition pH insensitive,

$$M_{+}^{-}(t) + R_{+}^{-}(t) = M_r^{-}(t) + R_r^{-}(t). \tag{14}$$

At the peak of $V_{+}(t)$ (t_1 = 1.2 ms for the cell shown in Figs. 1–3), the residual ERP [$V_r(t_1)$] had an average amplitude about equal to the noise of our recordings. Therefore, to a good approximation (combining Eqs. 12 and 14)

$$V_r(t_1) = 0 = M_{+}^{-}(t_1) + R_{+}^{-}(t_1). \tag{15}$$

Combining Eqs. 13 and 15 gives

$$M_{+}^{*}(t_1) = V_{+}(t_1), \tag{16}$$

i.e., the measured voltage at t_1 is a good measure of the metarhodopsin that can be affected by pH and no correction need be applied to the data in Fig. 3 *a*. These points are fit nicely over the entire pH range by the titration curve for a single site with pK 8.38.

A curious feature of Fig. 2 is that repeated exposures to pH-10.2 perfusate reduced all the ERP amplitudes, with the exception of the residual ERP, which remained unchanged. This would be just the sort of result expected if most of the visual pigment was accessible to the high pH solution and was slowly destroyed by it, whereas a small fraction of pigment, which gave rise to the residual ERP, was inaccessible to the solution and was not destroyed by it. It can be estimated that the inaccessible fraction

is ~20% of the total pigment by taking the ratio of the residual ERP to the large biphasic ERP at pH 7.4.

We wish to thank Gordon Fain, Alan Fein, and Marc Goldring for commenting on this manuscript.

This work was supported by National Institutes of Health grants EY-04196 to J. Lisman and EY-03104 to P. K. Brown.

Received for publication 9 January 1980.

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