Ionizable Groups and Conductances of the Rod Photoreceptor Membrane

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ABSTRACT The ionizable groups and conductances of the rod plasma membrane were studied by measuring membrane potential and input impedance with micropipettes that were placed in the rod outer segments. Reduction of the pH from 8.0 to 6.8 or from 7.8 to 7.3 resulted in membrane depolarization in the dark (by 2-3 mV) and an increased size of the light response (also by 2-3 mV). The dark depolarization was accompanied by an increased resting input impedance (by 11-35 MΩ). When the pH was decreased in a perfusate in which Cl$^-$ was replaced by isethionate, the membrane depolarized. When the pH was decreased in a perfusate in which Na$^+$ was replaced by choline, an increase of input impedance was observed (11-50 MΩ) even though a depolarization did not occur. These results are consistent with the interpretation that the effects of decreased extracellular pH result mainly from a decrease in rod membrane K$^+$ conductance that is presumably caused by protonation of ionizable groups having a pK_a between 7.3 and 7.8. Furthermore, from these results and results obtained by using CO_2 and NH_3 to affect specifically the internal pH of the cell, it seems unlikely that altered cytoplasmic [H$^+$] is a cytoplasmic messenger for excitation of the rod. When the rods were exposed to perfusate in which Na$^+$ was replaced by choline, the resting (dark) input impedance increased (by 26 MΩ $\pm$ 5 MΩ SE), and the light-induced changes in input impedance became undetectable. Replacement of Cl$^-$ by isethionate had no detectable effect on either the resting input impedance or the light-induced changes in input impedance. These results confirm previous findings that the primary effect of light is to decrease the membrane conductance to Na$^+$ and show that, if any other changes in conductance occur, they depend upon the change in Na$^+$ conductance. The results are consistent with the following relative resting conductances of the rod membrane: $G_{Na^+} \cong G_{K^+} > 2\text{-}5\ G_{Cl^-}$.

INTRODUCTION

The intermediate photoproducts produced after illumination of vertebrate rhodopsin involve a prominent series of ionization changes. In solutions of vertebrate visual pigment these ionization changes result in changes of pH (Radding and Wald, 1956; Erhardt et al., 1966; Ostroy, 1977). During the metarhodopsin I_{478} to metarhodopsin II_{380} reaction, protons are taken up by rhodopsin. In slightly buffered solutions this proton uptake leads to a less negatively charged protein, and usually to alkaline pH changes of the solution (Wong and Ostroy, 1973). Under the same conditions during the thermal decay of metarhodopsin II_{380}, protons are released, with the result that the rhodopsin

ionization is changed, and the pH changes of the solution are reversed (Ostroy, 1974). Light-induced pH changes have also been measured in suspensions of rod outer segments (McConnell et al., 1968; Emrich, 1971) and in perfused frog retinas (Ward and Ostroy, 1973). Furthermore, bacterio-rhodopsin is thought to effect a light-induced translocation of protons (Racker and Stoeckenius, 1974) which establishes a proton gradient across a membrane. Such proton gradients are thought to be the basis for energy transduction in several systems (Mitchell, 1956).

An ionizable group is one that is able to bind or release a proton, thereby altering the charge upon the group (Edsall and Wyman, 1958). The ionizable groups of the vertebrate photoreceptor membrane have not been characterized. However, in non-photoreceptor membranes ionizable groups affect ion permeabilities (Hille, 1968; Stephens, 1969). Also, the response properties of invertebrate photoreceptors are affected by changes in external pH (Stieve and Malinowska, 1974; Brown and Meech, 1975) and in internal buffering capacity (Coles and Brown, 1976). In invertebrate photoreceptors light-induced changes in intracellular pH also occur (Brown et al., 1976). Thus, the uptake of protons and their release from visual pigment may result in altered cytoplasmic pH, which may in turn result in altered ionization of chemical groups of the membrane; these events may play some part in the physiology of vertebrate photoreceptors.

Another question concerns the resting and light-induced conductances of the rod plasma membrane. A light-induced decrease in conductance to sodium ions is thought to cause the hyperpolarizing receptor potential, but it is not known if minor light-induced changes in other ionic conductances also occur (Toyoda et al., 1969; Penn and Hagins, 1969; Hagins et al., 1970; Korenbrot and Cone, 1972; Brown and Pinto, 1974; Werblin, 1975). An experiment to test for the existence of other light-induced conductance changes would be to measure the input impedance of a rod while the cell is bathed in Na^+-free perfusate. If light induces a change in conductance for only sodium, then light-induced changes in impedance should be abolished in Na^+-free perfusate (Goldman, 1943; Hodgkin et al., 1949). Also, at the present time information available from measurements of membrane potential does not allow one to calculate the resting Cl^- conductance. Reduction of $[Cl^-]_{out}$ does not cause a change in resting potential or response to light when the reduction is performed in 2–3 s, but one can not infer from this result that Cl^- conductance is low. Instead, it is possible that Cl^- conductance is high and that chloride ions equilibrate across the plasma membrane as fast as external chloride concentration is reduced (Brown and Pinto, 1974). We can estimate resting Cl^- conductance by measuring input impedance while bathing the rod in perfusate which contains reduced $[Cl^-]$.

The interpretation of measurements of membrane potential and input impedance must take into account the lateral interactions that have been demonstrated for the rods of many species. Because of these interactions, light captured in one outer segment affects not only its membrane potential, but also the membrane potential of neighboring rods (Fain, 1975). In both the toad (Fain et al., 1976) and the turtle (Copenhagen and Owen, 1976) the interactions are thought to be electrically mediated. Still another factor must be taken into

consideration for the interpretation of measurements of input impedance. This factor is the location of the recording micropipette. The inner and outer segments of the rod are connected by a modified cilium. Experiments with double barrel pipettes (in *Necturus*) show that the current-voltage relationship measured with the pipette in the outer segment differs from the relationship obtained when the pipette is located in the inner portion of the rod (Werblin, 1975). Thus, one would expect the measured impedance to depend upon the location of the measuring pipette.

This paper describes studies on the ionizable groups and conductances of the rod membrane with pipettes that were located in the rod outer segments. We studied only those cells that did not allow an appreciable fraction of the measuring current to spread to neighboring rods; this selection made easier the interpretation of the results we obtained. A preliminary abstract of some of the results has been published (Pinto and Ostroy, 1976).

METHODS

Hyperfine micropipettes filled with 4 M potassium acetate were used to make intracellular recordings from the outer segments of single rods in the isolated, perfused retina of *Bufo marinus* or the axolotl (*Ambystoma mexicanum*) (Brown and Pinto, 1974). Each retina was mounted receptor side up and placed on the stage of a compound microscope equipped with an infrared illuminator and image converters (Brown et al., 1977). At the beginning of each penetration the tip of the micropipette was positioned in the region of the tips of the rod outer segments; the pipette was then advanced (≤ 40 μm) in order to impale a rod. Because of this limited advancement we are certain that the pipette was able to impale only outer segments.

The following criteria were used in the selection of the data to be presented. (*a*) The plateau of the receptor potential had to exceed 8 mV for cells of *Bufo* and 5 mV for cells of the axolotl. (*b*) For experiments in which input impedance was measured the light-induced change in impedance had to exceed +8 MΩ. (*c*) The resting potential and light-induced change in potential had to agree within 1 mV before and after introduction of a test perfusate or, in the case of impedance measurements, within 10 MΩ.

Changes in input impedance were measured by passing a sinusoidally modulated current across the plasma membrane via the pipette (Pinto and Pak, 1974). This current had peak-to-peak amplitude of 0.5 nA and frequency between 10 and 30 Hz. A lock-in amplifier was used to detect the changes in both the component of voltage that was in-phase with the current and the component that lagged behind the current by 90° (quadrature component). The quadrature component was used for all experiments because it is unaffected by changes in the resistance of the pipette.

The interpretation of changes in input impedance depends on the spread of the measuring current from the impaled cell to its neighbors. If the current did not spread, then the impaled cell could be considered to be electrically isolated, and an increase in the input impedance would indicate an increase in the membrane resistance (Pinto and Pak, 1974). However, several experiments show that current spread between the inner segments of rods does occur (Copenhagen and Owen, 1976; Lamb and Simon, 1976). Therefore, we selected rods which gave large light-induced increases in input impedance because this selection excluded those cells for which a substantial fraction of the measuring current spread to the neighboring cells (see Discussion). Another obstacle to the interpretation of impedance measurements is the increase in membrane conductance elicited by hyperpolarization (Werblin, 1975; Schwartz, 1976). But our choice of cells with

large light-induced increases in input impedance also tended to select against cells for which this potential-induced change in conductance occurred.

The measurement of impedance was calibrated as follows. Assuming that the cells were electrically isolated, membrane time constant was measured (for five cells in *Bufo* and one cell in the axolotl). For *Bufo*, the mean time constant was 2.0 ± 0.3 ms SE; for the axolotl cell, the time constant was 5.5 ms. The calibration pulses that appear in the impedance records were generated by electronically inserting a parallel resistance-capacitance network in series with the impaled cell. The resistance of the network was 5 MΩ and the time constant was 2.3 ms. This time constant is approximately equal to that of the rod photoreceptor membrane (Pinto and Pak, 1974). Therefore in this paper the values for the measured changes in input impedance are given in terms of the change in membrane resistance that would have been required for an isolated cell to produce the observed change in impedance.

The composition of the normal perfusate for both species was Na^+ 111 mM, Cl^- 114.5 mM, K^+ 2.5 mM, $SO_4^=$ 0.6 mM, Ca^{++} 1.0 mM, Mg^{++} 1.6 mM, dextrose 5.6 mM. Unless otherwise noted, pH was 7.8; pH was buffered with 3 mM HEPES (N-2 hydroxyethylpiperazine N'-2-ethanesulfonic acid), a hydrogen ion buffer of pK_a = 7.5 and negligible affinity for Ca^{++} and Mg^{++} (Good et al., 1966). For the experiments in which external pH was varied, the pH was adjusted by titration of HEPES acid with varying amounts of NaOH; for these solutions the amount of NaCl was adjusted to keep [Na^+] constant. For low Na^+ perfusate (~2 mM), choline chloride was used to replace NaCl mole for mole.

For low Cl^- perfusate (~2 mM), sodium isethionate was used to replace NaCl mole for mole. Only a brief exposure (<5 min) to low Cl^- perfusate could be used reliably for each experiment because with longer exposures the membrane potential oscillated after offset of illumination. These oscillations were particularly noticeable for retinas that were several hours old. We have noticed similar oscillations in retinas that were bathed in low Ca^{++} perfusate and in retinas that were bathed in normal perfusate after pretreatment with low Na^+ perfusate made by replacing the NaCl with LiCl. We do not know the mechanism for the oscillations that occur under these various conditions, but it seems unlikely that each of these oscillations is due to the removal of a large shunting conductance that normally masks a potential-dependent conductance (see Quandt et al., 1977). In older preparations we sometimes also noticed steady changes in membrane potential and input impedance while bathing the retina in low Cl^- perfusate. These changes were most pronounced for recordings in which the amplitude of the receptor potential was small (≈5 mV) and from retinas that had received long (>10 min) cumulative exposure to low Cl^- perfusate. Because these effects were observed with poor impalements in older retinas they will be disregarded; the results we report for low Cl^- perfusate are from freshly isolated retinas (<1 h) that had received less than 10 min cumulative exposure to low Cl^- perfusate.

Perfusate with CO_2/HCO_3^- was made by substitution of 24 mM $NaHCO_3$ for NaCl and equilibration of the solution with a mixture of 5% CO_2-95% O_2. Perfusate with NH_3/NH_4^+ was made by substitution of 10 mM NH_4 Cl for NaCl; in this case the [NaCl] in the control perfusate was reduced by 10 mM and replaced by isosmotic substitution with sucrose. For perfusate with CO_2/HCO_3^- and its control, pH was adjusted to 7.8; for perfusate with NH_3/NH_4^+ and its control, pH was adjusted to 7.4. Changes in the composition of the contents of the perfusion dish took about 40 s to occur for the experiments in which pH was changed. For the experiments in which [Na^+] or [Cl^-] was lowered and for the experiments with CO_2/HCO_3^- and NH_3/NH_4^+ the perfusate flow was increased and these changes in composition were faster.

For six normal *Bufo marinus*, the mean pH of ventricular samples of whole blood, measured anaerobically, was 7.62 ± 0.015 S.E.

The stimulus was diffuse light of wavelength between 475 and 575 nm from a tungsten source with Bausch and Lomb 90-3-520 (Scientific Optical Products Div., Rochester, N.Y.) and Schott CG475 filters (Schott Optical Glass, Inc., Duryea, Pa.). The stimulus illuminance was between 2×10^{-9} and 5×10^{-8} W/cm².

RESULTS

Effects of Altered pH

When the pH of normal perfusate was lowered from 8.0 to 6.8, the rod plasma membrane depolarized in the dark; a comparable depolarization also occurred when pH was lowered from 7.8 to 7.3. This depolarization occurred for each of the nine cells studied in the axolotl, and for 11 of 13 cells studied in *Bufo*. For each of the above ranges of pH the magnitude of the depolarization was 2-3 mV. During illumination, the membrane hyperpolarized to the same absolute value of voltage whether the pH was high or low. Therefore, the responses to light became larger at the lower pH (Fig. 1a). For 5 of the 11 cells of *Bufo* that depolarized, the response kinetics were also altered: at the lower pH the membrane potential after stimulus offset returned more quickly than normal toward the resting value.

There was only a limited pH range over which changes in pH yielded the changes in response characteristics similar to those shown in Fig. 1. In one case (in the axolotl), when pH was reduced from 7.3 to 6.5 for 3 min, no change in resting potential or response occurred. However, irreversible gross disorientation of the outer segments was observed after this treatment; when the outer segments were disoriented, no further responses could be recorded from the
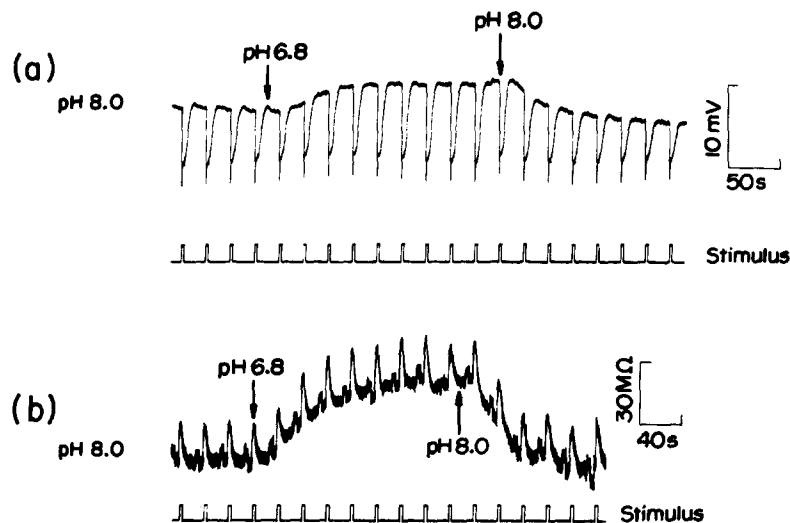

FIGURE 1. Altered external pH affects the membrane potential and input impedance of retinal rods of axolotl. (a) Upper trace, time-course of membrane potential. Lower trace, stimulus monitor. (b) Upper trace, time-course of input impedance. Lower trace, stimulus monitor. The small upward deflections preceding stimuli in (b) are calibration pulses (see Methods).

retina. When the pH of the perfusate was increased from 7.8 to 9.1, no effect upon photoreceptor resting potential or response to light was observed (for four cells in *Bufo*).

To explore the mechanism of the depolarization caused by decreasing the pH of the perfusate from 8.0 to 6.8, we measured membrane potential and input impedance under various conditions. The results of these changes are summarized in Table I. Decreasing pH from 8.0 to 6.8 caused an increase in resting input impedance (by 11–35 MΩ for five cells in the axolotl); at the lower pH the light-induced increases in impedance were about 25% larger (Fig. 1b).[1] In low Cl⁻ perfusate, reduction of pH from 8.0 to 6.8 caused a depolarization in the dark (for each of four *Bufo* cells). Three of these four cells exhibited an increase in the amplitude of the response to light at the lower pH; in addition, the response kinetics for these cells were altered at the lower pH: after offset of illumination the membrane potential returned to its resting value more quickly (Fig. 2). The results of an experiment in which the effects of altered pH were measured while the cell was bathed in low Na^+ perfusate are shown in Fig. 3. For this particular experiment the pH was increased (rather than decreased as for the above experiments) while the cell was bathed in low Na^+ perfusate. When the pH was increased in low Na^+ perfusate (for two cells in axolotl) the membrane potential did not change (Fig. 3a), but the input impedance did decrease by 11 MΩ for one cell and by 50 MΩ for the other cell (Fig. 3b). pH effects were observed in perfusates having calcium concentrations from 0.3–1.8 mM, but no systematic study of the dependence of the pH effects upon external calcium concentration was made.

All of the changes due to altered pH took about 40 s to reach completion, which is approximately as fast as the solution in the perfusion dish was changed, but this does not necessarily show that an ionizable group located on the outside of the plasma membrane is involved in the effects. To locate the ionizable group(s) we have made use of the finding that the internal pH of neurons can

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[1] Light-induced changes in input impedance were measured in normal perfusate for 89 cells of *Bufo* and 12 cells of the axolotl. For all of the axolotl cells and 74 of the *Bufo* cells, light induced an increase in input impedance (of between 2 and 30 MΩ). For the remaining 15 cells of *Bufo*, light induced a decrease in input impedance (of between 1 and 5 MΩ) during the initial portion of the impalement. Two further results indicate that the impedance decrease measured for the minority of cells was due to an increase in conductance that was elicited by hyperpolarization. (a) Four of the cells could be studied long enough to measure impedance several times: at the beginning of the experiment when the receptor potential was large, and later in the experiment when the receptor potential was smaller. The light-induced decrease in impedance was replaced by a light-induced increase in impedance as the magnitude of the receptor potential of each cell decreased. (b) Light-induced decreases in impedance were eliminated when the cells were bathed in low Na^+ perfusate (for three of the minority of cells), but were not eliminated by bathing the cells in low Cl⁻ perfusate (for four of the minority of cells). It is possible that, for this minority of cells, the pipette was placed in the inner rod, where light induces a decrease in resistance (Lasansky and Marchiafava, 1974), and hyperpolarization induces an increase in conductance (Werblin, 1975; Schwartz, 1976). It is also possible that the pipette was placed in the outer segment and responded to the sum of the light-induced decrease in Na^+ conductance plus an increase in conductance that was induced by hyperpolarization of some part(s) of the rod. The cells from which light-induced decreases in impedance were elicited were never sufficiently stable to be used for quantitative studies; therefore, these cells were not studied further.

be decreased or increased, without changing external pH, by bathing the neurons in perfusates which contain CO_2/HCO_3^- or NH_3/NH_4^+ (see Thomas, 1974). Bathing the retina briefly in perfusate that contained 10 mM NH_4Cl (but had constant $[Na^+]$, $[K^+]$, and pH) resulted in membrane depolarization (of 3 and 12 mV for two cells in *Bufo*) and an increase in the amplitude of the response to light (1 mV, 4 mV); bathing the retina briefly in perfusate that

TABLE I

SUMMARY OF RESULTS

Change of perfusate	Observed change in resting potential	Observed change in amplitude of light-induced hyperpolarization	Observed change in resting input impedance	Observed change in light-induced increase in input impedance	Other effects
pH 8.0 to 6.8 or pH 7.8 to 7.3	+2–3 mV	2–3 mV increase	+ 11–35 MΩ	From +15 MΩ (higher pH) to +20 MΩ (lower pH)	Faster return of membrane potential to baseline
pH 7.3 to 6.5	No change	No change	–	–	Disorientation of outer segments
pH 7.8 to 9.1	No change	No change	–	–	–
Normal $[Na^+]_o$ to low $[Na^+]_o$, pH = 8.0	−20 to −40 mV	Becomes undetectable	+15 to +30 MΩ	From +15 MΩ to 0 MΩ (undetectable)	–
pH 8.0 to 6.8 with low $[Na^+]_o$	No change	0 mV—no change	+11, +50 MΩ	0 MΩ—no change	–
Normal $[Cl^-]_o$ to low $[Cl^-]_o$, pH = 7.8	No change	No change	Undetectable change (<20%)	No change	Long term bathing in isethionate produces other effects (see Methods)
pH 8.0 to 7.0 with low $[Cl]_o$	+2 to +3 mV	2–3 mV increase	–	–	Faster return of membrane potential to baseline
$pCO_2 = 0$ to $pCO_2 = 40$ mmHg, pH = 7.8	−1 to −5 mV	1–3 mV increase	–	–	–
$[NH_4^+]_o = 0$ to $[NH_4^+]_o = 10$ mM, pH = 7.4	+3, +12 mV	1, 4 mV increase	–	–	Loss of transient undershoot

For resting potentials, + indicates depolarization. For input impedance, + indicates increase. See Methods for calculation of amplitude of input impedance.

contained 5% CO_2 (but had constant $[Na^+]$, $[K^+]$, and pH) resulted in membrane hyperpolarization (of 1–5 mV for three cells in *Bufo*) and an increase in the amplitude of the response to light (1–3 mV). All of these changes were reversible. From the above results it follows that presumed increases of internal pH, by the addition of ammonia or washout of carbon dioxide, were accompanied by depolarizations, whereas, from the previous results, increases of external pH produced hyperpolarizations. Attempts to alter intracellular pH by injecting H^+ iontophoretically (10^{-11} C of charge from a 1 M HCl pipette) yielded no discernible effects, probably because of the high buffering capacity of cytoplasm (Thomas, 1974).
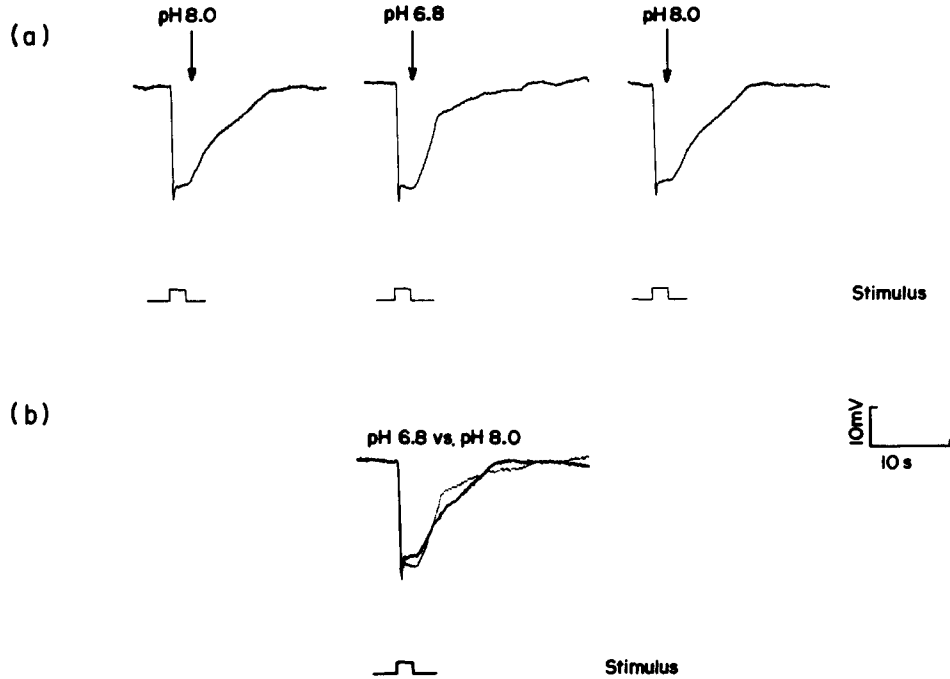
(a)

pH 8.0   pH 6.8   pH 8.0

Stimulus

(b)

pH 6.8 vs. pH 8.0

10 mV

10 s

Stimulus

FIGURE 2.   Altered external pH affects the membrane potential of a retinal rod of *Bufo marinus* that was bathed in low Cl⁻ perfusate. (*a*) Upper traces are responses to light, and lower traces indicate time-courses of stimuli. (b) Two responses from (*a*) are superimposed to illustrate a more rapid recovery of membrane potential to its resting value after stimulus offset.
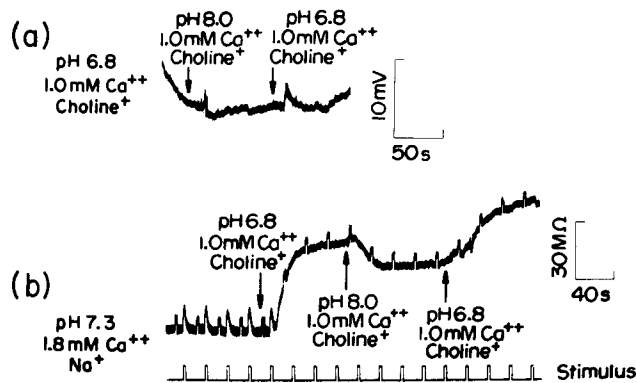
(a)   pH 8.0   pH 6.8
        1.0 mM Ca^{++} 1.0 mM Ca^{++}
        Choline^{+} Choline^{+}
   pH 6.8
   1.0 mM Ca^{++}
   Choline^{+}

   10 mV

   50 s

(b)   pH 6.8
        1.0 mM Ca^{++}
        Choline^{+}

                    pH 8.0   pH 6.8
                    1.0 mM Ca^{++} 1.0 mM Ca^{++}
                    Choline^{+} Choline^{+}
   pH 7.3
   1.8 mM Ca^{++}
   Na^{+}

   30 MΩ

   40 s

   Stimulus

FIGURE 3.   Effects of altered external pH upon rods of axolotl that were bathed in low Na^{+} perfusate. (*a*) Altered pH did not change membrane potential. The negative-going time-course at the beginning of (*a*) is due to the washout of normal perfusate that was in the perfusion dish before the beginning of the trace. (*b*) An increase of pH caused decreased input impedance (upper trace). Small upward deflections preceding stimuli are calibration pulses. Lower trace, stimulus monitor.

Photoreceptor Membrane Conductances

In an effort to estimate the relative ionic conductances of the rod membrane, input impedance was measured while the composition of the perfusate was altered. Only if a cell gave large (>10 MΩ) light-induced increases in input impedance did it also have stable resting input impedance.

Bathing the retina in low Na$^+$ perfusate resulted in an increased input impedance for eight cells in *Bufo* (mean 26 MΩ $\pm$ 5 MΩ SE) and two cells in the axolotl. The maximal increase in input impedance that could be elicited by light in normal perfusate was also measured for each of three cells. The maximal light-induced increase that we measured for a given cell in normal perfusate was between 25 and 100% of the increase in resting input impedance that we measured when that cell was bathed in low Na$^+$ perfusate. Light-induced increases in input impedance became undetectable in low Na$^+$ perfusate for all of these cells (detection limit — 10% of the light-induced increase that occurred in the normal perfusate).

We studied the Cl$^-$ conductance of the rod plasma membrane by measuring input impedance while bathing the rod in low Cl$^-$ perfusate. Only the results obtained from fresh retinas during brief exposures to low Cl$^-$ perfusate will be reported (see Methods). No detectable alteration in resting (dark) input impedance or in light-induced increases in input impedance occurred when the rods were bathed in low Cl$^-$ perfusate (for six cells in *Bufo*). An upper limit for the increase in input impedance that could have gone undetected when the retina was bathed in low Cl$^-$ perfusate was established in the following way, using four cells that we were able to study in both low Na$^+$ and low Cl$^-$ perfusates (Fig. 4). For each cell the average input impedance in the dark was measured before the retina was bathed in low Na$^+$ perfusate and after the low Na$^+$ perfusate was washed out of the perfusion dish. The difference between these two impedances is a measure of the long-term instability of the measurements over which we had no control. The long-term instability varied from cell to cell, and among the four cells the instability varied between 20 and 50% of the increase in impedance in the dark that occurred when the retina was bathed in low Na$^+$ perfusate. The change in average input impedance in the dark upon introduction and removal of the low Cl$^-$ perfusate was also measured for each of the four cells; in each case the latter change in impedance did not exceed the instability for that cell. Thus, the maximal change in input impedance that could have gone undetected when the retina was bathed in low Cl$^-$ perfusate was as little as 20% of the increase in impedance that occurred when the retina was bathed in low Na$^+$ perfusate.

DISCUSSION

Interpretation of Impedance Measurements

We measured input impedance of only those rods which produced a large light-induced increase in impedance. The purpose of this section is to show that this choice of cells tended to select against cells for which a substantial fraction of the measuring current spread from the impaled cell and also against cells for which

hyperpolarization induced a large increase in membrane conductance (Werblin, 1975).

The relationship between the measured input impedance and changes in the cell's membrane resistance will now be considered. Changes in the imaginary component of input impedance (proportional to changes in the quadrature component of voltage) were measured (see Methods). Let us first consider the
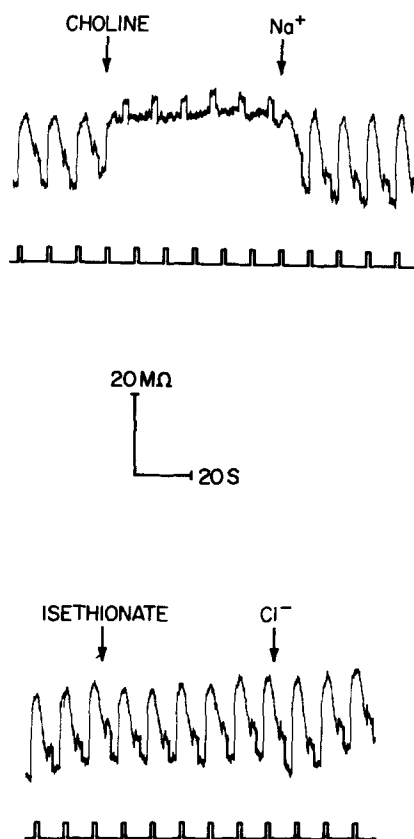

FIGURE 4. Input impedance is increased by bathing a *Bufo* rod in low Na^+ perfusate (top), but not by bathing the same rod in low Cl^- perfusate (bottom). Note also that light-induced changes in impedance were abolished in low Na^+ perfusate. The small upward deflections are calibration pulses. The stimulus monitor is beneath each impedance record.

case of a cell from which the measuring current spreads negligibly; in this case an increase in the imaginary component of impedance indicates an increase in the membrane resistance, because changes in membrane capacitance do not occur (Pinto and Park, 1974). Let us next consider the case where the measuring current does spread considerably from the impaled cell. In this case the imaginary component of input impedance will depend on rod-to-rod coupling resistance as well as membrane resistance. It is known that this second case does exist because current spread between rods has been demonstrated (Copenhagen

and Owen, 1976). Calculations of the input resistance (the input impedance for the application of direct current) have been made for networks that consist of cells between which current can flow (Minor and Maksimov, 1969; Jack et al., 1975; Lamb, 1976; Lamb and Simon, 1976). In most of these networks input resistance is directly proportional in coupling resistance, but large changes in membrane resistance do not produce large changes in input resistance. Similarly, large changes in membrane resistance would not be expected to produce large changes in input impedance. In our experiments changes in membrane resistance did produce large changes in input impedance for the cells that we selected (see Baylor et al., 1974, for resistance measurements from isolated photoreceptors). Therefore, the measuring current did not spread enough from the cells that we studied to make our measurements insensitive to changes in membrane resistance. There were two possible obstructions to current flow which could have been responsible for limiting the current spread in our experiments. First, the resistance of the connecting cilium could be high (see Werblin, 1975); secondly, rod-to-rod coupling resistance could be high. From the above evidence we conclude that the measurements of input impedance for the cells that we selected to study were biased in favor of being sensitive to changes in membrane resistance rather than to changes in coupling resistance.

Our choice also selected against cells for which hyperpolarization induced an increase in membrane conductance (see Werblin, 1975; Schwartz, 1976). Therefore, in the remainder of the Discussion it will be assumed that (a) measuring current did not spread significantly from the impaled cell, and (b) potential-induced changes in conductance did not occur in the dark as a result of changes in the perfusate.

Ionic Conductances of the Rod Membrane

The findings that light-induced increases in input impedance became undetectable in low Na^+ perfusate supports previous findings that the primary effect of light is to decrease the Na^+ conductance of the rod membrane (Toyoda et al., 1969; Penn and Hagins, 1969; Korenbrot and Cone, 1972; Brown and Pinto, 1974; Werblin, 1975). In low Na^+ perfusate light-induced changes in input impedance were undetectable. If these changes in impedance did occur, they had less than 10% of their normal amplitude. From this it follows that, if a change in conductance occurs in low Na^+ perfusate for ions other than Na^+, then the amplitude of this change must be less than 10% of the normal light-induced change of Na^+ conductance. Therefore, if any major changes in conductance occur for ions other than Na^+ in normal perfusate, these other changes depend on the change in Na^+ conductance.

Our results are consistent with the interpretation that the resting Cl^- conductance of the rod plasma membrane is lower than the resting Na^+ conductance. Bathing the cells in low Cl^- perfusate did not produce a detectable change in input impedance (input impedance did not change by more than 20–50% of the amount that the input impedance increased when the cell was bathed in low Na^+ perfusate). From this it follows that resting Na^+ conductance is at least 2–5 times greater than resting Cl^- conductance. We did not study the relative Na^+ and K^+ conductances, but none of our results are inconsistent with the previous

findings that these conductances are approximately equal (Toyoda et al., 1969; Werblin, 1975). In summary, we find the following relative resting conductances of the rod membrane: $G_{Na^+} \cong G_{K^+} > 2\text{--}5\ G_{Cl^-}$.

Mechanism for pH Effects

Decreasing the pH of the perfusate from 8.0 to 6.8, with normal [Na^+], produced the following effects: the membrane depolarized in the dark; the resting (dark) input impedance increased; and the light-induced changes in membrane potential and input impedance became larger. The finding that the depolarization (Fig. 1A) was accompanied by an increased input impedance (Fig. 1B) is consistent with the interpretation that the depolarization results from a decrease in membrane conductance for an ion that has an equilibrium potential more negative than the rod's resting potential. The two ions that might have appropriate equilibrium potentials are K^+ and Cl^-, but there are two reasons to believe that Cl^- ions are not involved. If Cl^- were important, the changes in potential and impedance would not occur in low Cl^- perfusate. But pH-induced alterations in membrane potential, amplitude, and time-course were observed in low Cl^- perfusate (Fig. 3). In addition, we have found that the resting Cl^- conductance of the rod is no more than 20–50% of the resting Na^+ conductance. Therefore, a further reduction of the Cl^- conductance elicited by decreased pH probably could not markedly decrease total membrane conductance. Also, the following results obtained in low Na^+ perfusate suggest that a decrease in pH does decrease membrane conductance for K^+. The membrane potential probably lies close to the K^+ equilibrium potential when the membrane is bathed in low Na^+ perfusate. When the membrane potential lies close to the K^+ equilibrium potential, a decreased membrane K^+ conductance would not be expected to alter the membrane potential, but it would be expected to increase input impedance. Indeed, decreasing the pH did not alter membrane potential in low Na^+ perfusate, but it did increase input impedance. Thus, the changes in resting potential and resting input impedance are consistent with the interpretation that decreased pH results in decreased membrane conductance to K^+ ions.

The amplitude of the light-induced increases in input impedance became larger when pH was decreased (see Fig. 1B). These larger light-induced increases in input impedance can also be explained by decreased membrane K^+ conductance. For a cell in which Na^+ and K^+ conductances are the largest, the input resistance (changes of which will give proportional changes of input impedance for the measuring frequencies that were used, see Pinto and Pak, 1974) is given by the formula for the parallel combination of the two conductances:

$$R_{in} = \frac{R_{Na} \cdot R_K}{R_{Na} + R_K},$$
(1)

where R_{in} = input resistance, R_{Na} = 1/Na^+ conductance, and R_K = 1/K^+ conductance. Differentiating with respect to R_{Na} shows that a given light-induced change in R_{Na} will produce larger changes in R_{in} as R_K is made larger:

$$\frac{dR_{in}}{dR_{Na}} = \left[\frac{R_K}{R_{Na} + R_K}\right]^2.$$ (2)

Thus, the observation that light-induced increases in impedance became larger when pH was lowered (see Fig. 1B) can be explained by a pH-induced decrease in membrane conductance to K^+.

The amplitude of the light-induced hyperpolarization increased when pH was decrased (see Fig. 1A). However, we cannot tell whether this increase in the amplitude of the light-induced hyperpolarization can also be explained by a decrease in membrane K^+ conductance. For a cell whose membrane can be modeled by a two-branch electrical circuit, membrane potential is given by:

$$V_m = \frac{E_{Na} \cdot R_K + E_K \cdot R_{Na}}{R_{Na} + R_K},$$ (3)

where V_m = membrane potential, E_{Na} = Na^+ equilibrium potential, and E_K = K^+ equilibrium potential. Differentiating with respect to R_{Na} shows that a given change in R_{Na} will produce changes in V_m that are maximal when $R_{Na} = R_K$, and minimal when either $R_K \gg R_{Na}$ or $R_{Na} \gg R_K$:

$$\frac{dV_m}{dR_{Na}} = \frac{R_K(E_K - E_{Na})}{(R_{Na} + R_K)^2}.$$ (4)

Thus, a decrease in K^+ conductance will cause increased amplitude of the light-induced changes in V_m if and only if the resting Na^+ conductance is lower than resting K^+ conductance. We cannot measure the specific ionic conductances accurately enough to tell whether this condition was met. Thus, we cannot say whether an increase in the amplitude of the light-induced change in membrane potential would be expected to result from a pH-induced decrease in membrane K^+ conductance.

In summary, the observed increases in resting and light-induced changes in input impedance, and the depolarization in the dark can be explained by a decrease in membrane K^+ conductance evoked by decreasing pH from 8.0 to 6.8. However, we do not know the basis for the pH-induced changes in receptor potential amplitude and time course.

One may now ask whether pH-induced changes in membrane conductances for ions other than K^+ also occurred. If the only change when pH is decreased is a decrease of membrane K^+ conductance, then the membrane potential during illumination should be less hyperpolarized when the retina is bathed in perfusate of decreased pH than when bathed in perfusate of normal pH. However, the absolute value of the membrane potential during illumination was nearly constant, regardless of pH. Thus, it is possible that changes in membrane conductance for ions other than K^+ occur when pH is changed.

An alternative explanation for the above pH effects is that they might have been caused by an increase in rod-to-rod coupling resistance. If one assumes that impalement of an outer segment by a micropipette damages the outer segment, then at rest the damaged rod will be depolarized with respect to its neighboring unimpaled rods. The currents that flow through the conducting pathways between rods will tend to keep the impaled rod hyperpolarized. An

increase in the resistance of the conducting pathways will decrease the currents that help maintain the hyperpolarization of the impaled cell. This increase in coupling resistance will increase the input impedance of the impaled rod, whereas the decrease in current will further depolarize its membrane. A reduced amplitude of the light-induced changes of both membrane potential and input impedance would also be expected to occur. However, the responses were made larger when pH was decreased. It also follows that a pH-induced increase in rod-to-rod coupling resistance would depolarize damaged cells that are studied in low Na^+ perfusate. However, a depolarization did not occur when the pH of low Na^+ perfusate was lowered. Thus, we think that the pH effects could not have resulted solely from an increase in rod-to-rod coupling resistance.

A related alternative explanation is that the pH effects were due to an increase in the resistance of the connection between inner and outer segments of the rod. It is possible that the outer segment of the cell is damaged during impalement and that current flows through the connection. This current will tend to maintain the hyperpolarization of the outer segment. If the resistance of the connection is increased, then the current will be diminished. This increase in resistance will cause an increase in both the resting input impedance and the light-induced change in input impedance of the outer segment, and the decreased current will cause the outer segment membrane to depolarize. These latter changes were in fact what we observed. However, if this alternative explanation were the basis for the pH effects, then we would have observed the largest effects during the worst impalements. Inasmuch as this was not the case, we do not believe that an increase in the resistance of the connection between inner and outer segments could have been the sole cause of the pH effects.

We did not study the mechanism for the pH-induced alteration in the time-course of the response to light that occurred for some of the cells of *Bufo*. However, these changes cannot be explained by a simple time-invariant change in K^+ conductance.

In summary, we conclude that decreased external pH results in decreased G_{K^+}. This decreased G_{K^+} in turn causes increased resting input impedance, increased amplitude of the light-induced changes of input impedance, and membrane depolarization in the dark. However, we do not know if decreased external pH also alters other ionic conductances, and we do not know the basis for the pH-induced changes in the amplitude and time course of the receptor potential.

Properties of the Ionizable Groups

The effects observed in perfusate of low pH are consistent with a pH-induced decrease in membrane conductance to K^+ (see above). The pK_a of the groups that are responsible for the decrease in conductance might be determined by measuring the pH effects at many different pH's for one cell. However, we were not able to study any one cell at many values of pH. Therefore, we estimated the pK_a of the ionizable groups from the following more crude information. The pH-induced changes in resting potential were undetectable in the range 6.5–7.3 and in the range 7.8–9.1. However, effects of approximately equal magnitude were seen when pH was changed either from 7.3 to 7.8 or

from 6.8 to 8.0. Hence, the pK_a of the titratable group probably lies between 7.3 and 7.8, which is consistent with the value of 7.6 obtained by Gedney and Ostroy (1973). It is of interest to note that protonation of the postulated group causes decreased conductance for a cation; the same type of behavior also occurs for barnacle muscle fiber (Hagiwara et al., 1968).

The location of the ionizable groups that were affected by the changes of pH of the perfusate cannot be specified with the present data. However, our data suggest that the sites are exposed to the outside of the plasma membrane. HEPES is a large, zwitterionic, and presumably impermeable buffer (Good et al., 1966), and protons alone are not permeable across the membranes of structures such as chloroplasts and mitochondria (Mitchell, 1966). The changes in membrane potential and input impedance observed in our experiments occurred as rapidly as the pH changes of the perfusate. Also, the experiments using ammonia and carbon dioxide to affect only the internal pH of the cell gave changes of membrane potential that were opposite to the change in potential that resulted from altered pH of the perfusate. It is interesting to note that if the site of the ionizable groups were the outside of the plasma membrane of rod outer segments, then the formation of the disk from an invagination of the plasma membrane (Young, 1967) would also place such ionizable groups inside the disk membrane.

From our experiments it seems unlikely that altered cytoplasmic $[H^+]$ serves as a cytoplasmic messenger for carrying excitation from the disks to the plasma membrane. Light induces a decrease in Na^+ conductance of the plasma membrane that results in a hyperpolarization. However, changes in external pH primarily affected the membrane conductance for K^+. Also, although bathing the retina in CO_2/HCO_3^- or NH_3/NH_4^+ to change the internal pH gave alterations in membrane potential and response to light, these alterations, except for one cell, were smaller than the light response. These data indicate that changing internal pH may have affected certain ionizable groups. However, the small size of the effects observed suggests that these groups do not play a major role in the generation of the light-induced electrical response. In particular, we tested the effect of the increased cytoplasmic pH that could have resulted from proton uptake during the metarhodopsin I_{478} to metarhodopsin II_{380} reaction. When cytoplasmic pH was presumably increased by bathing the retina in perfusate that contained NH_3/NH_4^+, the membrane depolarized, rather than mimicked the hyperpolarization observed upon illumination. Thus, it appears unlikely that altered cytoplasmic $[H^+]$ mediates the light response of rods.

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