Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 1892–1896, April 1983
Biophysics

Protons block the dark current of isolated retinal rods

(visual transduction/membrane current/calcium ions/light effect)

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Communicated by Britton Chance, December 30, 1982

ABSTRACT **Membrane currents of isolated frog rods were recorded with the suction pipette technique and tested by perfusion techniques for their sensitivity to H^+. The following facts have been established. (*i*) Increased $[H^+]$ suppresses the Na^+ conductance of the outer segment rapidly and reversibly. (*ii*) H^+ acts in the rod interior. (*iii*) The $[H^+]$ necessary to cause a 50% decrement in Na^+ conductance is inversely related to the $[Ca^{2+}]$ over 5 orders of magnitude. (*iv*) The sensitivity to H^+ and the sensitivity to light, as a function of $[Ca^{2+}]$, have the same slope. Thus, H^+ act like light in effecting membrane current suppression but behave as if their effect is mediated through Ca^{2+}. Based on these results and properties of rod disk membrane phosphodiesterase, we propose that protons produced in the light-activated hydrolysis of cGMP liberate Ca^{2+} from the disks by ion exchange.**

In rod outer segments of vertebrates, light absorption causes a decrease in plasma membrane Na^+ conductance (1). Because the rhodopsin molecules that absorb light are embedded in a stack of membrane disks not connected with the plasma membrane (2, 3), it is generally assumed that one or more transmitter substances mediate between photon absorption and decrement in Na^+ conductance. The facts that elevated intracellular $[Ca^{2+}]$ decreases the Na^+ conductance (1, 4–6) and that illumination causes release of Ca^{2+} from rods into the extracellular space (7, 8) support the hypotheses (9) that light effects the release of Ca^{2+} from the intradiskal space and that Ca^{2+} is the intracellular transmitter for excitation. However, the process by which a single photon triggers the release of 10^3–10^4 Ca^{2+} ions (5, 7, 8) has not yet been identified.

The absorption of a photon by rhodopsin enables it to convert several hundred molecules of GTP-binding protein into a form (10) that activates membrane-bound phosphodiesterase (PDEase) (11–13). Activated PDEase catalyzes the hydrolysis of cGMP with a turnover estimated for an amphibian rod under physiological conditions of $>10^4$ cGMP molecules per absorbed photon within 1 sec (14–16). The hydrolysis reaction cGMP → 5′-GMP stoichiometrically liberates a H^+ over a pH range that likely includes the physiological pH of the rod cytoplasm (the reaction exposes a phosphoryl group of pK = 6.3), and the production of H^+ has been used to determine the kinetics of rod PDEase activation (12) and inactivation (17) in *in vitro* measurements of rod membranes. Certainly, if PDEase activation proceeds with the rate *in vivo* that can be estimated from *in vitro* data, many thousands of H^+ per photon would be produced. In a frog rod typical of our preparation, with a 35-μm outer segment containing 1,200 disks (cytosol vol, ≈0.5 pl), a flash of 200 photons that would completely block the Na^+ conductance should produce $>10^6$ H^+ within 1 sec (16). Thus, a considerable number of H^+ may be available during the light response to displace cations bound as counterions to fixed charges at the outer or inner surfaces of the disk membranes. A fraction of the bound cations might be Ca^{2+}. This report describes the effects of extra- and intracellular changes of pH on the dark currents of isolated rods. The results suggest that H^+ may act as an intermediary between cGMP hydrolysis and Ca^{2+} release.

MATERIALS AND METHODS

Rods from freshly dissected dark-adapted *Rana pipiens* retinae were isolated under IR observation by gentle brushing into Ringer's solution (normally, 84 mM NaCl/24 mM $NaHCO_3$/2.5 mM KCl/1.0 mM $CaCl_2$/1.0 mM $MgCl_2$/1.2 mM $MgSO_4$/5 mM glucose/0.2% bovine serum albumin). Unless otherwise stated, the solutions were saturated with 95% O_2/5% CO_2 prior to the addition of bovine serum albumin, and kept in hermetically sealed vials. A significant fraction of the rods retained their ellipsoids in the isolation procedure (see Fig. 1*A*); the ellipsoid, a 15-μm region lying just under the outer segment and connected to it by a ciliary neck, contains a dense mass of mitochondria that supplies the ATP needed to maintain the dark current and for other metabolic processes of the cell. Cells that have ellipsoids maintain normal membrane currents and light response kinetics for well over an hour. The rod membrane current was measured with the suction pipette technique (18, 19). Individual outer segments with ellipsoids (hereafter called "rods") were sucked ellipsoid-in into a pipette projecting into a chamber on an inverted microscope stage, as shown in Fig. 1*A*. The pipette had a 6-μm constriction near the entrance, providing a partial seal of 6–10 MΩ around the rod. A current-to-voltage transducer (voltage clamp circuit) measured at zero potential difference the external longitudinal current flowing between the ellipsoid inside the pipette and the outer segment protruding into the chamber. Electrical currents were recorded on magnetic tape at a bandwidth of 0–30 Hz.

A separate perfusion pipette having a 10- to 20-μm-diameter orifice was placed ≈20 μm from the rod, permitting rapid changes of the solution around the rod. The fluid in the chamber was stirred by blowing a stream of moist 95% O_2/5% CO_2 (or pure O_2) onto the surface. Perfusion was visually monitored by means of 1-μm latex beads added to the perfusates. Perfusion with a solution of altered ionic composition generated junction currents that could be minimized with minor (≤1%) adjustments of the ionic strength of the perfusate. Control experiments showed that such adjustments had no effect on the fractional suppression of dark current by low pH perfusion. Tests monitoring the bead flow and appearance of junction potentials showed that the solution surrounding the rod could be changed within 0.5 sec. Control experiments in which Na^+-free perfusate, high KCl perfusate, or altered $[Ca^{2+}]$ were used showed that the system was capable of completely altering the solution around the outer segment within less than 1 sec and of maintaining the altered state. The maximum total perfusate ex-

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Abbreviation: PDEase, phosphodiesterase.

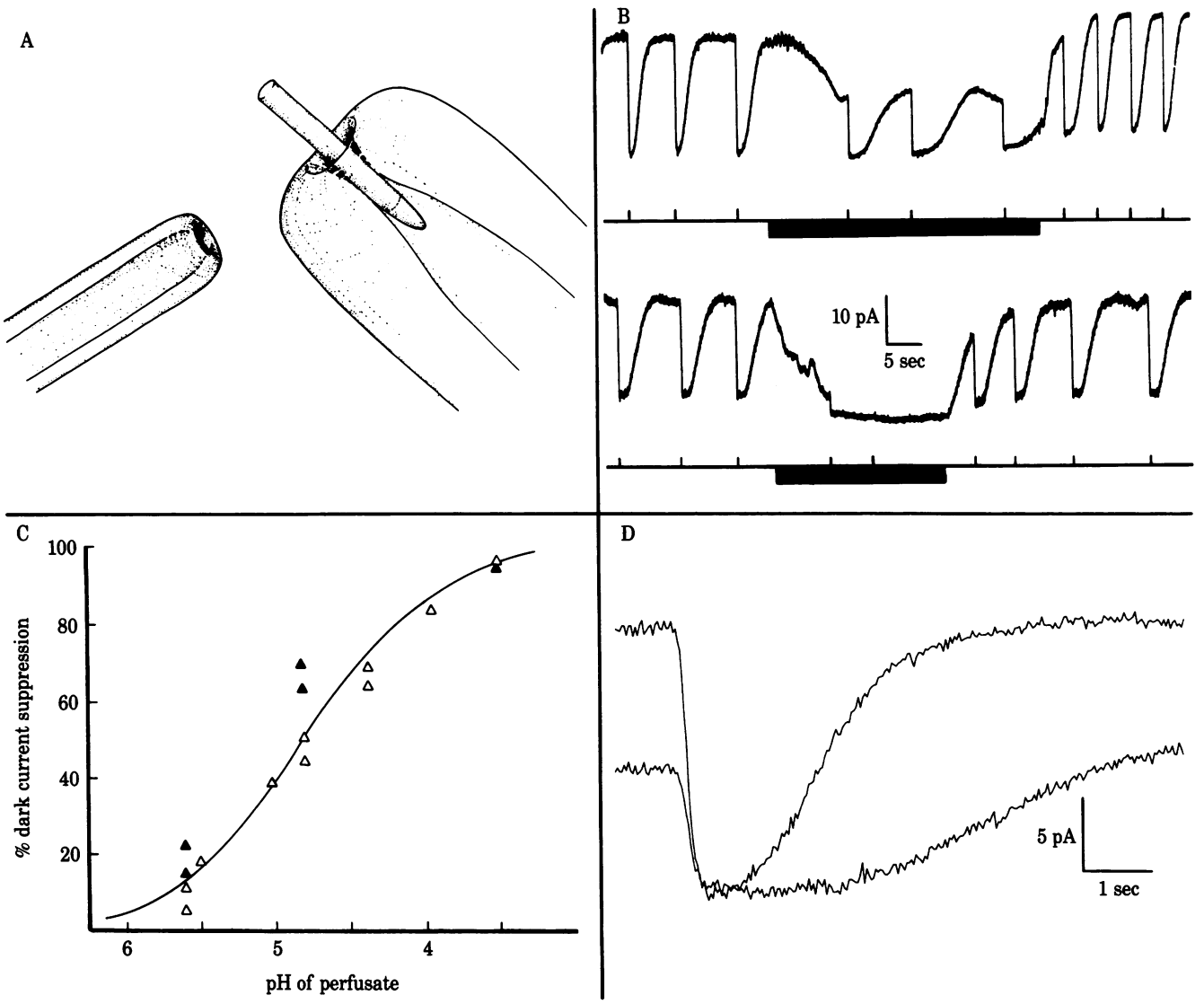

FIG. 1. Tracing from a videotape record of an experiment. (*A*) Method used for recording membrane currents of solitary rod outer segments with ellipsoids. The rod is held in a suction pipette with the ellipsoid projecting inward; the pipette projects into a 1.5-mm-thick chamber (300-μl vol) mounted on an inverted microscope stage. The pipette is connected via a salt bridge and a Ag/AgCl electrode to an amplifier that maintains a voltage clamp across the seal resistance, which is typically 6–10 MΩ; the resistance without the cell in the pipette is $\approx$1 MΩ. The perfusion pipette shown on the left is used to apply a test solution to the outer segment of the rod. All experiments are monitored with an IR video system. The diameter of the rod outer segment is 6 μm. (*B*) Membrane currents from rods responding to 20-msec flashes (at the times indicated by the tic marks) that yield about 300 isomerizations. Heavy bars indicate time of perfusion. The upper trace is a record of a rod perfused with pH 4.8 Ringer's solution, which causes $\approx$50% suppression of the dark current (i.e., the light-suppressible membrane current); in the lower trace, a rod is perfused at pH 3.5, and the dark current is completely suppressed. Small junction currents, which offset the saturated response level, are seen in both cases. Recovery from these relatively short-duration perfusions can occur in <1 sec, and the cycle of suppression and recovery can be repeated many times on an individual cell. (*C*) Relationship between the pH of the perfusion solution and the dark current suppression in Ringer's bicarbonate containing 1 mM Ca^{2+}. Each symbol represents an experiment in which a cell was perfused at the indicated pH for at least 1 min and from which the cell completely recovered its preperfusion dark current and light response kinetics. Different cells were used for experiments at different pH values; different symbols at a given perfusate pH represent different cells. The curve has the form for titration of a weak acid (pK_a = 4.8). (*D*) Two saturating responses of a rod to flashes of 250 isomerizations. The larger response occurred in normal Ringer's solution (pH 7.4). The smaller response, a few seconds later, occurred during perfusion at pH 4.6. Note the extended plateau phase of the response at low pH and the retardation of the relaxation.

truded in an experiment never exceeded $\approx$0.01% of the chamber volume (300 μl), and so the application of negative pressure to the perfusion pipette at the end of a perfusion period combined with the continual stirring produced by the blower were capable of rapidly returning the cell to the baseline condition. Monochromatic 500-nm flashes of calibrated intensity, duration (usually 20 msec), and area were focused on the rod.

RESULTS

Protons Block the Dark Current. When the pH of the Ringer's solution around the outer segment is suddenly lowered, the light-suppressible dark current is rapidly diminished. Traces from two different rods exposed to perfusates at pH 4.8 or 3.5 are shown in Fig. 1*B*: at pH 4.8, the dark current is approximately half suppressed whereas at pH 3.5, there is complete suppression. Both the diminishment of the dark current and the recovery from the H^+-induced suppression can occur in <1 sec. The way in which the fractional suppression of dark current depends on the pH of the perfusate in bicarbonate Ringer's solution with 1 mM Ca^{2+} activity is shown in Fig. 1*C*: the effect has an apparent pK of $\approx$4.8.

A lowered pH that produces partial suppression of the dark

current lengthens the saturated light response time, both extending the duration of the plateau and increasing the time constant of the relaxation, as shown in Fig. 1*D*. In addition, lowered pH increases the light sensitivity (the fraction of the maximal photocurrent suppressed by a single photon), increasing the relative (and at some pH values even the absolute) amplitude of low-light-level responses and shifting the half-maximal point of the response vs. light intensity curve to lower intensities. Raising the external pH to >8 has the opposite effect: the dark current (i.e., maximal photocurrent) increases, the saturated response time is accelerated, and the low-level-light response is decreased in amplitude, shifting the half-maximal point of the response vs. light intensity curve to higher intensities. Over the pH range from 3.5 to 10.5, these perfusion effects of tens of seconds duration are rapidly reversible.

The Decrease in Dark Current Is Due To Blocking of the Na^+ Conductance. Suppression of the dark current by lowered pH could result from either a decrease in the Na^+ conductance, the dominant membrane permeability of the outer segment, or an increase in the outer segment K^+ conductance. To discriminate between these effects, we performed experiments similar to those used to show that elevated [Ca^{2+}] blocks the Na^+ conductance (1). After baseline measurements were taken, a cell was gently pushed out of the suction pipette and the ellipsoid was saturated with 200 μM ouabain to completely inhibit the Na^+/K^+ pump. The cell was then sucked back into the pipette and the gradual decline of the dark current was monitored by giving occasional saturating flashes. Temporary exposure to either light or H^+ sufficient to shut off the dark current greatly retarded the decay of the dark current, and removal of either the light or the H^+ reversed the effect. Decrease in the rate of decay of the dark current would be expected if the Na^+ influx were reduced but not if the K^+ efflux were increased. The retardation of the decay of the isolated receptor potential of ouabain-poisoned rods by acid perfusion, previously noted in whole retina (20), is consistent with our observations on isolated rods. Also qualitatively consistent is the finding that H^+ block the reswelling of isolated outer segments hyperosmotically shocked with NaCl (21).

The Site of Action of H^+ Is Intracellular. Experiments of the sort shown in Fig. 1 cannot determine whether the site of the action of H^+ is inside or outside the plasma membrane. To distinguish between these possibilities, we used methods based on transmembrane diffusion of CO_2 and NH_3, which liberate H^+ intracellularly without altering the extracellular pH, as shown directly with intracellular pH microelectrodes (22, 23). When a cell is externally perfused with NH_4Cl, NH_3 rapidly enters and is protonated, raising the internal pH [for example, from 7.4 to 8.0 in barnacle muscle (24)]. As NH_4^+ enters more slowly through ionic channels and is deprotonated, the internal pH tends to return to normal. When the NH_4Cl-containing perfusate is removed, the reverse effect occurs: NH_3 diffuses out, leaving behind an excess of H^+, and the pH undershoots [from somewhat <8 to 6.7 in barnacle muscle (24)].

The effect on rod photocurrent of 1 min of perfusion with Ringer's solution containing 50 mM NH_4Cl is shown in Fig. 2*A*. During perfusion, the maximal photocurrent increases to $\approx$1.5 times its resting value; a similar (though not so large) increase occurs when the external pH is raised to 9. When the perfusate is removed, there is a transient decrease in photocurrent that is maximal about 30 sec after the return to normal Ringer's solution and is followed by a slow recovery of the preperfusion response. The time course of these effects is similar to the time course of directly measured intracellular pH changes (24).

A block of the light response is also observed when a rod that has been kept in CO_2-free Ringer's phosphate at pH 7.5 is suddenly exposed to bicarbonate Ringer's solution that has been saturated with 5% CO_2 (Fig. 2*B*). In this case, H^+ are liberated intracellularly as CO_2 diffuses into the cell, is hydrated to carbonic acid, and partially dissociates to HCO_3^- and H^+ (22–24). The effects of CO_2 perfusion and the recovery from NH_4Cl perfusion on the dark current and response kinetics are the same as the effects observed with external acid perfusion, and we conclude that the suppression of dark current by H^+ is due to their action in the cell interior. They may also, however, act on sites on the exterior of the plasma membrane.

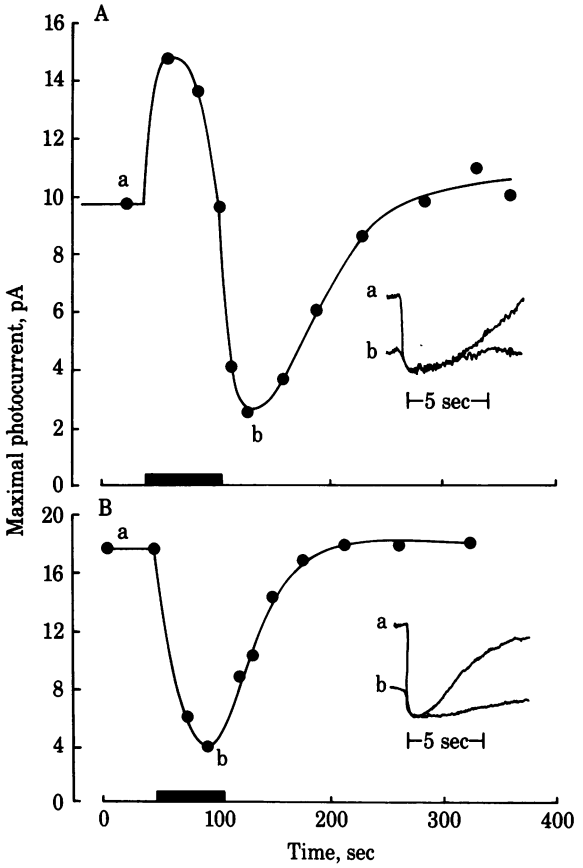

FIG. 2. (*A*) Time course of recovery of the maximal photocurrent of a rod measured with a flash of 300 isomerizations, during and after perfusion with 50 mM NH_4Cl. Prior to perfusion, the cell was bathed in Ringer's solution containing 50 mM choline chloride (essentially the same results were observed when choline chloride was not used). During the period marked by the heavy bar, the cell was perfused with Ringer's solution containing 50 mM NH_4Cl at the identical pH (7.4). (*Inset*) Traces of the responses of the cell prior to perfusion (a) and near the minimum in the recovery curve (b). (*B*) Suppression of the dark current by perfusion with CO_2. During the period marked by the heavy bar, Ringer's solution containing 50 mM $NaHCO_3$ and saturated with 5% CO_2 was substituted for normal Ringer's solution; both the external pH and the [Na^+] were kept constant. (*Inset*) As in *A*.

The Sensitivity to H^+ Is Diminished by Lowering the Ca^{2+} Activity. The immediate effect of exposing rods to lowered Ca^{2+} activity is a transient increase in the dark currents and photocurrents (25, 26). Rods exposed for minutes to solutions of lowered Ca^{2+} activity exhibit a greatly diminished sensitivity to light (25, 27, 28). Whereas a rod in (normal) 1 mM Ca^{2+} might have a light intensity vs. response amplitude curve with a half-maximal point at 30–50 photons, the half-maximal point of the intensity–response curve for a rod exposed for 5 min to 1–10 nM Ca^{2+} may shift to more than 10^4 photons. Within the framework of the Ca^{2+} hypothesis, this diminished light sensitivity

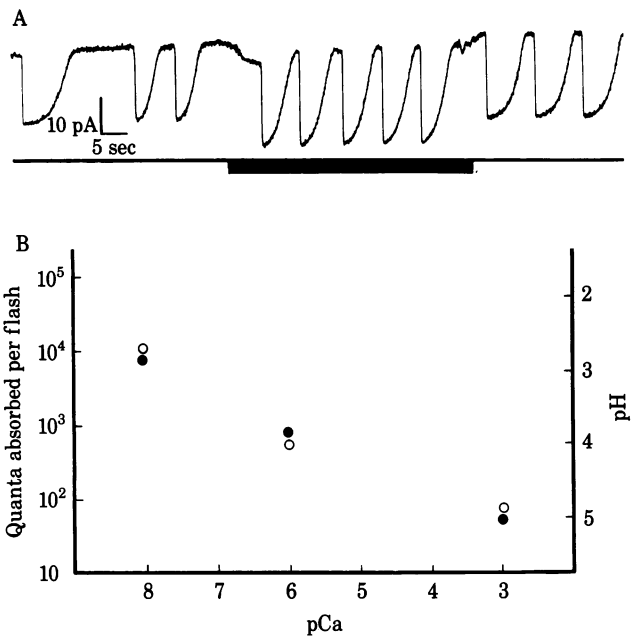

FIG. 3. (*A*) Responses of a cell in Ringer's solution containing 10 nM Ca^{2+} before, during, and after perfusion at pH 3.5. The initial flash produced 120,000 photoisomerizations; each subsequent flash, 40,000. At this low Ca^{2+} activity, the low pH perfusion causes no decrement in maximal photocurrent (actually an increase occurs during the perfusion). (*B*) Relationship between Ca^{2+} activity, light sensitivity, and H^+ sensitivity of rod membrane current. Both light sensitivity and H^+ sensitivity covary with Ca^{2+} activity. Results represent amount of light (○) or H^+ (●) for 50% suppression of dark current; each point, except the pH sensitivity point at the lowest Ca^{2+} activity, is based on a titration curve such as given in Fig. 1*C*. The slope of the pH vs. pCa curve is roughly −2.

has been interpreted to mean that the internal store of light-releasable transmitter has been diminished by exposure to low Ca^{2+} medium (25). Another interpretation of the large light sensitivity shifts in low Ca^{2+} is that the cytoplasmic (as opposed to intradiskal) bound Ca^{2+} is greatly diminished, resulting in a much greater Ca^{2+} buffering capacity (28). In either interpretation, less Ca^{2+} is available per isomerization to reduce the Na^+ conductance. If the action of light generates H^+ that release Ca^{2+} by ion exchange from binding sites within the disks or in the cytoplasmic space, then a decrease in sensitivity to light produced by lowering the Ca^{2+} activity of the Ringer's solution should be paralleled by a decrease in the sensitivity to H^+.

The responses of a rod that had been incubated for ≈0.5 hr in Ringer's solution containing 1 μM Ca^{2+} and then in 2 mM EGTA (estimated Ca^{2+} activity, 10 nM) for 10–15 min before beginning recording are shown in Fig. 3. Perfusion with pH 3.5 Ringer's solution, which, at normal Ca^{2+} activity, completely suppresses the dark current, did not diminish the dark current at all (nor did perfusion of other cells in low Ca^{2+} with CO_2, as in Fig. 2*B*). The relationship between the pH that effects a 50% suppression of the dark current and the Ca^{2+} activity of the Ringer's solution in the chamber is given in Fig. 3*B*. Each of the points in this figure represents a complete light or pH titration (obtained from several cells), such as that shown in Fig. 1*C*. Also given in the figure is the approximate number of absorbed photons required to half suppress the dark current under the same conditions. We note that the relationships between pCa and light sensitivity and between pCa and the sensitivity to H^+ have about the same slopes.

DISCUSSION

Our experiments show that exogenous H^+ can suppress rod membrane current, that this suppression can occur without change in external pH, and that sensitivity to H^+ (like light sensitivity) depends on Ca^{2+} activity. If light generates H^+ in the outer segment in the quantity and at the rate that they are supplied in these experiments, then protons are a link in photoexcitation, most likely exerting their effect through Ca^{2+}. Our present results, however, do not permit unambiguous calculation of the number of H^+ introduced into the outer segment to suppress the dark current either with NH_4^+ or CO_2 perfusion or with changes in external pH. For example, the number of H^+ introduced by CO_2 perfusion depends on, among other things, the initial internal HCO_3^- concentration and whether or not carbamate formation occurs; this information, requisite to performing the appropriate calculations, is not available.

Calculations based on *in vitro* assays (16), adjusted to account for intracellular physiological conditions in intact rods, suggest that a light flash of 200 isomerizations, which will completely suppress the dark current of a rod under normal conditions, will produce 10^6–10^7 H^+ within 1 sec. However, an increase in intracellular H^+ activity in response to light has not yet been measured. But even at light intensities that cause maximal PDEase activation in the cell, the rate of H^+ generation may be lower than the rate of the hypothesized H^+/Ca^{2+} exchange. Such rapid exchange, aided by the restricted diffusion space between adjacent disks, together with binding at other buffer sites, might cause H^+ to disappear as fast as they are generated, rendering it difficult to measure light-induced H^+ production in the intact cell directly.

It can be argued that the intracellular buffering capacity of the rod outer segment is so high that the number of released H^+ available for exchange is too small to account for the reported release of ≈10^4 Ca^{2+} per isomerization from amphibian rods (7). This argument is valid only if the H^+ affinities of buffering sites not complexed with Ca^{2+} are equal to or higher than the H^+ affinity of sites binding Ca^{2+}. If the converse were true, H^+ would preferentially go to sites binding Ca^{2+}, effecting release. Rod outer segments contain 0.1–3 mol of Ca^{2+}/mol of rhodopsin, most of which appears bound within the disks (29, 30). Outer segments with leaky plasma membrane are able to maintain millimolar [Ca^{2+}] inside the disks in the presence of <10 μM Ca^{2+} in the external medium (31). Ion exchange experiments show that Ca^{2+} can be displaced from the disk interior by monovalent and divalent cations (30–32) and H^+/Ca^{2+} exchange in both directions has been reported (32). One possible mechanism for Ca^{2+} release would involve entry of H^+ into the disks via a transmembrane H^+/Ca^{2+} exchange and subsequent displacement by these H^+ of Ca^{2+} ions associated with lipids or proteins on the inner surface of the disk membrane. Evidence has been presented for a H^+/Ca^{2+} exchange mechanism in reconstituted vesicles made from whole disks and lipids (33), but the identity of the exchanger is unknown. Rhodopsin, when incorporated into lipid bilayers, becomes specifically conductive for H^+ on illumination (34). The conductance per molecule is small (≈10 fS) but potentially could be part of a Ca^{2+}/H^+ exchange. Our own unpublished ^{45}Ca experiments with broken rods show that ≈10 H^+ are needed to release one Ca^{2+} and that the overall cation selectivity is $H^+ > Ca^{2+} > Na^+ > K^+ > Mg^{2+}$. In intact rods, such exchange would be expected to be more efficient because proton production takes place in the small interdisk space, leading to high local concentrations, whereas our fractured rod preparation contains some residual cell fragments and proteins that contribute significant buffering capacity.

As pointed out to us by D. A. Baylor (personal communi-

cation), some of our data also could be interpreted in terms of a blocking effect of H^+ on the Na^+/Ca^{2+} exchange mechanism that is apparently responsible for the extrusion of Ca^{2+} from the rods in response to light (7, 8). If an inward leak of Ca^{2+} in the dark is balanced by extrusion via a Na^+/Ca^{2+} exchanger, then blocking the exchanger with H^+ would result in a net influx of Ca^{2+}, shutting down the Na^+ conductance. The retardation of the recovery phase of the light response under low pH conditions would result because the Ca^{2+} released inside the rod by light cannot be extruded at the normal rate. In low Ca^{2+} solutions, H^+ would be relatively ineffective in reducing the dark current, because little or no Ca^{2+} is leaking into the cell in the dark. However, the recovery phase of the light response during low pH perfusion should be as much retarded in low as in normal Ca^{2+} solutions. For a given fractional Na^+ conductance decrease, the light-induced rise in internal free Ca^{2+} must reach approximately the same level independent of external Ca^{2+} activity. Therefore, the extrusion of Ca^{2+} by the exchanger should still be slowed by H^+; this is not observed (Fig. 3*A*). Additional *ad hoc* assumptions relating the sensitivity of the Na^+/Ca^{2+} exchange to pH as a function of Ca^{2+} activity would have to be made to explain the ineffectiveness of protons in low Ca^{2+} solutions.

The H^+/Ca^{2+} exchange hypothesis proposed here *per se* provides no explanation for the depolarizing effects of intracellular injections of cGMP on rod membrane potentials (35–37) nor do our experiments speak to the issue. However, the proposal that protons produced by cGMP hydrolysis act as an intermediate messenger in excitation may provide perspective on failure to observe rapid light-induced changes in cGMP in the rod (38–40). For if the production of H^+, and not the decrement in [cGMP], is the relevant intermediary in the chain of events leading to Na^+ conductance decrease, [cGMP] need not decrease much in response to light. In fact, what ideally would be expected on the basis of a H^+/Ca^{2+} exchange hypothesis is that rod guanylate cyclase would be able to replace cGMP as fast as it is hydrolyzed by PDEase. Data on rod guanylate cyclase activity (41–43) support the view that, at the normal GTP and Ca^{2+} levels in the cytosol, the frog rod could produce $\geq 10^7$ cGMP molecules per sec.

We thank P. A. Liebman, W. H. Miller, W. Englander, and D. Baylor for helpful comments and discussion. This work was supported by National Institutes of Health Grants EY-02660, EY-00102, and GM-25256 and Center Grant EY-01583.

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