Ionic Blockage of the Light-Regulated Sodium Channels in Isolated Rod Outer Segments

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ABSTRACT   We have investigated, with osmotic techniques, the light-regulated Na^+ channels in rod outer segments (ROS) and ROS fragments freshly isolated from the frog retina. Values of Na^+ permeability (P_{Na}) similar to those observed electrophysiologically in the retina were observed using the osmotic technique (continuous flow) described by Korenbrot and Cone. In the other osmotic techniques that we explored, P_{Na} was greatly diminished, if not completely suppressed; however, we found with these techniques that antioxidant conditions (N_2 atmosphere or EDTA) significantly increased P_{Na}, suggesting that the Na^+ channels are highly sensitive to membrane oxidation. Using the continuous flow technique, we investigated the H^+ and Ca^{++} dependence of the Na^+ channels and found that both of these ions, at micromolar activities, can block the channels. Raising the external H^+ activity decreases P_{Na} (reversibly) in a single "sigmoidal" response with an apparent pK_a of 5.8. Similarly, in the presence of the ionophores X537A or A23187 which allow equilibration of Ca^{++} across membranes, the Na^+ channels are blocked when the external Ca^{++} activity is increased from 10^{-7} to 10^{-5} M. This high sensitivity to both H^+ and Ca^{++} ions suggests that high field strength anionic sites may exist in or near the Na^+ channels and that the channels are blocked when these sites bind H^+ or Ca^{++} ions.

INTRODUCTION

The mechanism by which rhodopsin regulates the Na^+ channels in the plasma membrane of visual receptors is unknown. It seems likely that cytoplasmic ions such as H^+ and Ca^{++} may play an important role in the regulation mechanism. In vertebrate rods, a steady Na^+ current flows between the outer and inner segments of the receptor cell (Hagins et al., 1970; Hagins, 1972; Penn and Hagins, 1972). In the dark, the outer segments are highly permeable to Na^+, and the Na^+ current entering the outer segment is large (Sillman et al., 1969; Yoshikami and Hagins, 1970; Korenbrot and Cone, 1972; Zuckerman, 1971, 1973). Light initiates a process (involving an unknown cytoplasmic transmitter) which blocks the Na^+ channels (Baylor and Fuortes, 1970; Hagins, 1972; Cone, 1973). This markedly reduces the Na^+ current entering the outer segment and hyperpolarizes the receptor (Penn and Hagins, 1969; Hagins et al., 1970; Zuckerman, 1971; Werblin, 1975). This hyperpolarization diminishes the contin-

uous release of transmitter from the synaptic end of the receptor (see e.g., Schacher et al., 1976).

In this investigation, both the H^+ sensitivity and the Ca^{++} sensitivity of the light-regulated Na^+ channels were studied to clarify the characteristics of these channels, as well as to help identify the cytoplasmic transmitter which regulates them.

Although the H^+ sensitivity of the Na^+ channels has not been directly studied, the H^+ dependence of the late receptor potential (LRP) has been investigated in frog retinas. Sillman et al. (1972) found a sigmoidal relationship between pH and LRP amplitude, which they assumed was due to variations in the visual pigment kinetics. Ostroy and his colleagues found that the LRP decreased in amplitude as external pH was lowered from 9 to 6.4, but they found a nonsigmoidal relationship (Ward and Ostroy, 1972; Gedney and Ostroy, 1974). They suggested that the pH changes associated with the metarhodopsin I to metarhodopsin II reaction oppose the generation of the LRP. Thus, investigations to date have not revealed the H^+ sensitivity of the Na^+ channels.

Yoshikami and Hagins (1971) have postulated that the cytoplasmic transmitter released by light is Ca^{++}. According to their hypothesis, Ca^{++} is actively pumped out of the cytoplasm, resulting in low cytoplasmic Ca^{++} activity in the dark. Thus, a rhodopsin molecule, on absorbing a photon, could release Ca^{++} ions from the disc into the cytoplasm. The Ca^{++} ions would then diffuse to and block the Na^+ channels in the plasma membrane. The Na^+ channels would reopen when the excess Ca^{++} ions were removed by the pumping process. This Ca^{++} hypothesis has been given suggestive support by a variety of recent experiments (see Discussion). It should be noted that, in addition to Ca^{++}, cyclic GMP is also a possible candidate for the internal transmitter (see e.g., Fletcher and Chader, 1976).

Much of the electrophysiological work on the effects of H^+ and Ca^{++} ions is difficult to interpret because the site of action is unknown: the observed effects on the dark current and on the LRP could be caused by changes in either the outer segment or the inner segment of the photoreceptor. Fortunately, the Na^+ channels in the plasma membrane of the rod outer segment (ROS) can be studied directly by physically isolating the ROS from the rest of the cell. This eliminates effects due to the rest of the cell. The ionic permeability of the isolated ROS is easily studied with osmotic techniques. Using a continuous flow osmotic technique to examine the permeability of freshly isolated outer segments, Korenbrot and Cone (1972) showed that Na^+ rapidly enters the ROS in the dark and that illumination markedly reduces the Na^+ influx. Moreover, with this technique, the light-regulated Na^+ flux is in good quantitative agreement with electrophysiological data. Using different osmotic techniques, Bownds et al. (1973, 1974) and Brodie and Bownds (1976) found qualitatively comparable results, whereas other investigators have not (Cobbs and Hagins, 1974; Chabre and Cavaggioni, 1975). Therefore, we examined a variety of osmotic techniques.

A preliminary report of this investigation has already appeared (Wormington and Cone, 1975).

MATERIALS AND METHODS

Preparation

Adult grass frogs (*Rana pipiens*, southern variety), 3–4 inches in body length, were used in all experiments. The frogs were kept in constant darkness and killed by decapitation under dim red light. The eyes were then left in complete darkness for ≅5 min. At the end of this time the eyes were opened, and the retinas were gently dissected free under infrared (IR) light. This procedure took ≅2.5 min.

The ionic flux through the plasma membrane of the ROS was observed by measuring the rate at which outer segments recovered in volume after an osmotic shock. In most experiments, this was accomplished with a simple continuous flow apparatus and flash-microphotography.

Continuous Flow Technique

The continuous flow apparatus was similar to the one used by Korenbrot and Cone (1972). A "T" junction mixing chamber was formed by drilling three channels in a lucite block. A suspension of outer segments flowed into the junction through one arm of the T, while a hyperosmotic solution flowed in through the opposed arm. These two solutions mixed at the junction and then flowed up through the third channel, the "mixing channel," to an exit point on the surface of the block. With the aid of dyes, Korenbrot and Cone (1972) observed good mixing in their apparatus. Similarly, we also found mixing was essentially complete shortly after the two solutions combined at the T junction. However, it is important to note that improper positioning of the ends of the tubing in the two arms of the T junction may result in inadequate mixing. The mixing channel was 1 cm in length and 0.1 cm in diameter. After leaving the mixing channel, the outer segments flowed along a duct made by cutting a 2.0-mm-wide slit in a single layer of Parafilm (about 115 μm thick; American Can Co., Neenah, Wis.). The duct was covered with a 22 × 60-mm glass cover slip that was clamped to the lucite block. The entire assembly was mounted on a microscope stage, and a ×10 objective was focused at the exit point of the mixing channel. The outer segments were flash-photographed using a 35-mm microscope camera (Miranda Mixax Laborec, Animco, Silver Spring, Md.) mounted on the microscope with a ×10 ocular. Deep red or IR light was used to photograph the outer segments. An extremely high speed, panchromatic film with extended sensitivity in the red (Kodak 2475 recording film; Eastman Kodak Co., Rochester, N. Y.) was used for most experiments. For a few experiments Kodak high speed IR film was used in conjunction with a Corning 2540 filter (λ > 880 nm; Corning Glass Works, Science Products Div., Corning, N. Y.) over the light source condenser. Photographs were made using a Xenon flash tube with a 0.1-ms duration (Cosco Megastrobe, MS = 200 E, Edmund Scientific Co., Barrington, N. J.) filtered through a Schott RG 659 (3 mm) filter (Schott und Gen., Jena, W. Germany) which passed light with λ > 680 nm. Focusing was accomplished using an IR viewer (Varo, Inc., Garland, Texas). The photonegatives were projected onto a large drawing board where the lengths of the outer segments were measured using calipers.

The suspension of outer segments was prepared under IR light using an IR viewer (Find-R-Scope, FJW Industries, Mt. Prospect, Ill.). The light source was a PR-2 flashlight bulb filtered either through a Corning 2540 glass filter (3.8 mm) which passed light with λ > 880 nm or through two 3-mm Schott RG 1000 filters which passed light with λ > 920 nm. The isolated retina was gently shaken in a loading well which was sealed immediately after withdrawing the retina. The loading well was a 60-μl chamber drilled in a lucite block and was connected to the mixing channel by a 25-cm-long polyethylene tubing

(inner diameter 380 μm). The loading well was also connected to a plastic 1-ml syringe containing standard saline solution that was used to drive the outer segments into the mixing channel. The hyperosmotic test solution was stored in a second 1-ml syringe, also connected to the mixing channel through polyethylene tubing. Hence, when both syringes were driven at the same rate, equal volumes of both solutions were mixed. A constant speed motor drove the plungers of both syringes, either together or independently. The rate determined the time after shock at which the outer segments were flashphotographed as they emerged from the mixing channel. All experiments were performed at 21–24°C, within 3 min after the retina was shaken in the loading well.

During the experiments, all photographs were taken as the outer segments emerged from the exit hole of the mixing channel. At the beginning of each experiment, only the syringe containing standard saline was driven, in order to obtain three photographs of unshocked (control) outer segments. The remaining outer segments in the loading well were then osmotically shocked in the mixing channel by driving both syringes. Usually four photographs of these shocked outer segments were obtained. Thus, in each experiment, shocked outer segments could be compared to unshocked outer segments from the same suspension. A new ROS suspension was used for each of the three time points investigated (2.9, 6.4, and 9.7 s after shock). The 2.9- and 9.7-s intervals were chosen because Korenbrot and Cone (1972) showed the rate of volume recovery is essentially constant over the interval from 2.5 to 10 s. The constancy of the recovery rate was checked in most experiments by making additional observations at 6.4 s. In the results reported here, the recovery rate was calculated from the slope defined by the 2.9- and 9.7-s observations. To insure that proper comparisons were made, in many experiments every outer segment and fragment in each photograph was measured, and histograms of the length distributions were compared. However, this was both tedious and time consuming, so for all other experiments only 8–12% of the outer segments in each flash-photograph was measured, selecting the longest outer segments present. The measurements were made without the observer knowing the experimental conditions under which each photograph was obtained. For the results reported in this paper, the lengths of >66,000 outer segments were measured. In each experiment the lengths of 20–80 control (unshocked) outer segments and 60–100 shocked outer segments were measured, and the ratio of the arithmetic averages of each group was determined. The validity of this method was examined in 85 experiments, in which complete histograms were compiled after completing the short (10%) analyses (see Results). Complete histograms of the length distributions were prepared by measuring every outer segment and fragment in each photograph. For each time point, photographs of both shocked and unshocked outer segments from the same suspension were measured.

To insure that the dimensions of the mixing channel did not affect the results, another version of the apparatus was constructed. A mixing channel 9 times longer and 3 times narrower giving 20 times more shear gave the same results as the original version, indicating that dimensions and shear were not critical.

Other Techniques

In an attempt to follow the osmotic behavior of single outer segments with time, two additional techniques were developed. The first technique (the "glass slide technique") involved gently dabbing the retina into 20 μl of standard saline solution in a small well on a glass slide. The wells were made by cutting holes in vinyl plastic electrical tape (Scotch 33+, 3M Co., St. Paul, Minn.) layered on the glass slide. A 10-μl aliquot of this outer segment suspension was then transferred to 10 μl of hyperosmotic "test" solution. Cover slips were placed over the wells, and the slide was mounted on the microscope stage.

Flash photographs using IR light could then be taken at various times after a shock. A variation of this technique was used for the oxidation experiments. Instead of taking photographs of the same outer segments sequentially in time, the outer segments were left in the dark 3–5 min after the shock, and photographs were taken at the end of this time. In some of these experiments isolated outer segments were bleached just before the osmotic shock.

The second technique (the "Nuclepore filter technique") involved dabbing the retina on a Nuclepore membrane filter (Nuclepore Corp., Pleasanton, Calif.) stretched over a 3-mm-wide channel cut in vinyl plastic tape (two thicknesses) layered on a glass slide. Another glass slide was lowered onto the Nuclepore filter. Between this glass slide and the filter was a small piece of cover glass (3 mm × 2 mm × 150 μm) attached to the glass slide and centered over the channel. The distance between the small piece of cover glass and the Nuclepore filter, on which the outer segments rested, was $\cong$20 μm. The microscope was focused on the outer segments contained in this small volume. The Nuclepore filter was 11-μm-thick and contained cylindrical pores about 8 μm in diameter at a density of about 10^5 pores/cm^2. To shock the outer segments, the hyperosmotic solutions were driven through the wide channel underneath the filter. Outer segments shrank with a half-time of $\cong$3 s in this apparatus because the thickness of the Nuclepore filter and the distance between the cover glass and the filter was small, keeping diffusion times to a minimum.

Bleaching Exposure

The bleaching light was a single flash from an M-3 flashbulb (about 30-ms duration) filtered through a Schott KG-3, Wratten 8, and Schott BG-18 filter combination (Schott KG-3 and BG-18; Schott und Gen.; Wratten 8; Eastman Kodak Co.). This combination passed light between 480 and 600 nm. Within the experimental limits of resolution, no volume changes were induced by flash illumination alone. The outer segments suspended in the loading well were bleached 2–3 min before osmotic shock. The control photographs for each experiment were obtained after this bleach.

The bleaching light source was calibrated using solubilized rhodopsin solutions under conditions identical with those of the experiment (except for the solubilization of the rhodopsin). A single flash was found to bleach more than 50% of the pigment.

Total Light Exposure during Preparation

The IR light source used for dissection of the retina and preparation of the ROS suspension was calibrated using a psychophysical technique. A white surface was placed at the position of the retina during the dissection. The IR light reflected from the white surface was below visual threshold for one observer and just about threshold for another observer. The IR source was then replaced by a white light source which was calibrated in terms of the rate at which it bleached solubilized rhodopsin. Neutral density filters were added to the white light source to make the light reflected from the surface match visually the intensity of reflected IR light.

Outer segments were exposed to the IR light for <2.5 min during the entire preparative procedure. After being isolated in the loading well, the outer segments were in the dark until flash-photographed. Assuming there are 7.5 × 10^5 rhodopsin molecules/disc (Liebman, 1973), <0.75 rhodopsin molecules/disc were bleached using the Corning 2540 filter. With the RG 1000 filters, <0.02 rhodopsin molecules/disc were bleached.

These total light exposures before the osmotic shock are orders of magnitude less than the "dim red light" exposure described by Korenbrot and Cone (1972). Significantly, despite the great differences in light exposures during the different preparative

procedures, the rate of volume recovery after NaCl shocks in the dark did not appear to be affected; that is, the exposures did not appear to "light-adapt" the outer segment.

Solutions

All solutions were prepared in distilled, demineralized water with reagent grade salts. The concentrations of hyperosmotic solutions are expressed in units of isoosmotic pressure (1 U [1 Is] is 240 mosM). Test solutions were prepared by adding salts to the standard saline solution (Table I). The concentrations required to produce the appropri-

TABLE I

SOLUTIONS

	NaCl	KCl	CaCl₂	GG	P	MES	Glycerol	NaAc	KAc	CaAc₂	EGTA
	mM	mM	mM	mM	mM	mM	mM	mM	mM	mM	mM
GGP saline	115	2.5	2	4	4						
6 Is NaCl	789	2.5	2	4	4						
6 Is glycerol	115	2.5	2	4	4		1073				
Ac GGP saline				4	4*			115	2.5	2	
6 Is NaAc				4	4*			735	2.5	2	
MES saline	115	2.5	2			10					
6 Is NaCl	789	2.5	2			10					
10⁻⁶ M Ca⁺⁺ saline‡	115	2.5	0.1	4	4						0.08
6 Is NaCl	789	2.5	0.1	4	4						0.08
10⁻⁷ M Ca⁺⁺ saline‡	115	2.5	1	4	4						1.34
6 Is NaCl	789	2.5	1	4	4						1.34
0.5 mM EGTA	115	2.5		4	4						0.5
saline											0.5
6 Is NaCl	789	2.5		4	4						0.5

	NaNO₃	KNO₃	Ca (NO₃)₂	CaEDTA
	mM	mM	mM	mM
NO₃ saline	115	2.5	2	
6 Is aNO₃	825	2.5	2	
3 mM CaEDTA saline				3

Abbreviations used in this table: GG, glycylglycine; P, piperazine dihydrochloride; MES, 2(N-morpholino)ethane sulfonic acid; Ac, acetate; EGTA, ethyleneglycol bis (β-aminoethylether)-N,N' tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

* Piperazine used instead of piperazine dihydrochloride.

‡ Ca⁺⁺ activities were calculated using an apparent dissociation constant for EGTA of 10⁻⁷·⁴⁷⁵ at pH 7.4.

ate osmotic pressures were determined either from published freezing-point-depression data (Weast, 1967) or from actual osmotic pressure measurements using a freezing-point-depression osmometer (Knauer Semimicro, type M, Ted Pella, Inc., Tustin, Calif.). Except where indicated, all solutions were buffered at pH 7.4. The fluorochrome, N,N'-didansyl cystine (DDC) was obtained from Sigma Chemical Co. (St. Louis, Mo.).

The ionophores X537A (lasalocid) and A23187 were used in some experiments to increase the permeability of the outer segment to Ca⁺⁺ ions. X537A was obtained as a gift from Dr. Julius Berger (Hoffman-La Roche, Inc., Nutley, N.J.), and A23187 was obtained as a gift from Dr. Robert L. Hamill (Eli Lilly and Co., Indianapolis, Ind.). The ionophores were first dissolved in 95% ethanol. A small aliquot of this stock solution was added to the physiological saline solution immediately before use. The maximum

concentration of ethanol in the saline solutions was always <0.03%. Control experiments in which ethanol without the ionophore was added to the saline solution demonstrated that ethanol by itself had no effect. The concentration of the ionophore was checked spectrophotometrically.

RESULTS

Behavior in KCl and NaCl

Korenbrot and Cone (1972) investigated the permeability of the plasma membrane of isolated rod outer segments to both KCl and NaCl. We have confirmed their results using various modifications of their continuous flow technique. Outer segments shocked with hyperosmotic KCl responded as shown by the squares in Fig. 1. The outer segments rapidly shrank to a stable volume. This behavior is characteristic of an osmometer placed in a solution made hyperosmotic by addition of an impermeant solute. Flash illumination before the
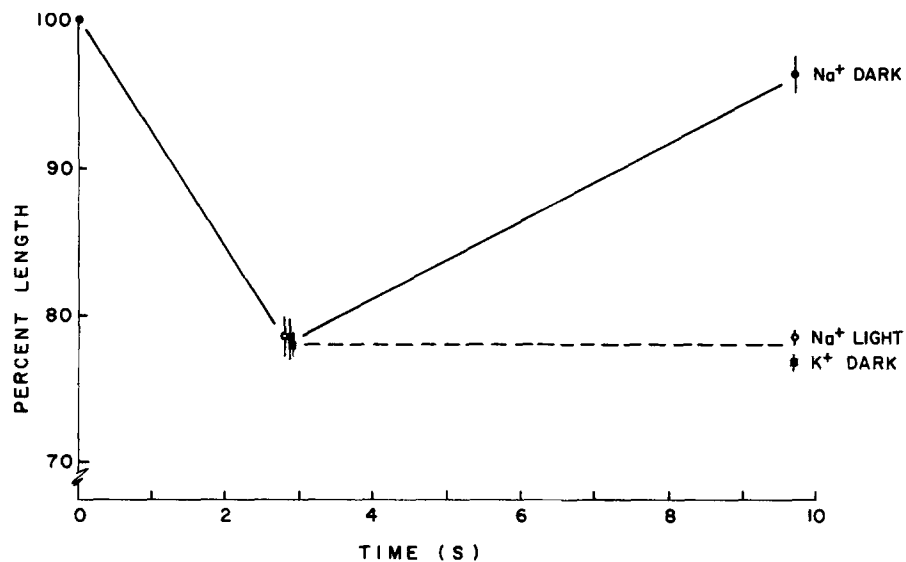
Figure 1. Osmotic behavior of outer segments after a 3.5-Is NaCl shock in the dark (●) or after a bleach (O) and a 3.5-Is KCl shock in the dark (■). For the hyperosmotic shocks reported in this paper, the length of the outer segment decreases, but its width remains nearly constant (Korenbrot and Cone, 1972). Thus, measurements of lengths are sufficient to determine volume changes. The solid line depicting the volume recovery after the NaCl shock in the dark is drawn with a slope of 2.6% pre-shock volume/s. For the NaCl shock, the volume in the dark at 2.9 and 9.7 s after the shock was 78.5±1.5% (4) and 96.4±1.3% (6), respectively, and 78.5±1.5% (4) and 78.6±0.5% (3), respectively, for outer segments bleached before the shock (mean±SD). For the KCl shock the volume at 2.9 and 9.7 s after the shock was 78.0±0.9% (3) and 76.8±0.7% (5), respectively. Numbers in parentheses indicate number of separate experiments performed; over 100 outer segments were measured in each experiment.

osmotic shock does not alter this behavior of the outer segment (Korenbrot and Cone, 1972).

For the hyperosmotic shocks reported in this paper, the length of the outer segment decreases but its width remains nearly constant (Korenbrot and Cone, 1972). Thus, measurements of lengths are sufficient to determine volume changes.

The response to a hyperosmotic NaCl shock is shown by the circles in Fig. 1. The outer segments shrank rapidly to the same volume as with the equivalent KCl shock. Bleached outer segments remained at this volume. However, dark-adapted outer segments shocked in NaCl slowly recovered in volume. The recovery of the outer segment is characteristic of an osmometer shocked with a permeant solute (NaCl).

Recovery to normal volume after the hyperosmotic shock with the permeant solute (NaCl) must be due to an internal expansion force. As discussed by Korenbrot and Cone, this internal expansion force reverses direction if the outer segment swells beyond normal size. Under the conditions of the osmotic technique, the rate of volume recovery $(\frac{dv}{dt})$ after a hyperosmotic NaCl shock is the product of the Na^+ permeability (P_{Na}) of the plasma membrane and the area (A) across which the flux of Na^+ occurs. Hence, the permeability to Na^+ is

$$P_{Na} = \frac{\dfrac{dv}{dt}}{A}.$$

Korenbrot and Cone have presented evidence that outer segments are permeable to both Na^+ and Cl^- and that light regulates the influx of Na^+, not Cl^-. They report that after a Na_2SO_4 shock, no detectable volume recovery occurred in either the dark or the light. Thus, the outer segments are impermeable to Na_2SO_4, even though permeable to NaCl. This result indicates that the plasma membrane must be permeable to both the cation and the anion if volume recovery is to occur. Korenbrot and Cone also found that, in the light, outer segments do not recover their volume in either $NaNO_3$ or NaCl, but, in the dark, they recover their volume in $NaNO_3$ at a rate similar to that in NaCl. This result suggested that light controlled the influx of Na^+ but not Cl^-, in that it is unlikely that both the NO_3^- and Cl^- ions enter the outer segment through the same light-regulated channel at the same rate.

Subsequently, Cobbs and Hagins (1974) reported that outer segments hyperosmotically shocked with NaCl do not recover in volume either in the light or in the dark under the conditions of their experiments. They explained this result by assuming the outer segments are not permeable to Cl^-. In contrast, Zuckerman (1973) concluded on the basis of extracellular electrophysiological recordings that the outer segments are permeable to Cl^-.

Since the Cl^- permeability of the outer segments is an important electrophysiological parameter, we have further examined the conclusion by Korenbrot and Cone that Na^+ permeability, and not Cl^- permeability, limits the rate of volume recovery in both light and dark. To do this we investigated volume

recovery rates in a variety of salts in which the cation, or the anion, or both, were in equilibrium with uncharged, lipid-soluble molecules. It has been known for some time that NH_3 and acetic acid (HAc) are lipid soluble and can easily cross cell membranes (Jacobs, 1940). In addition, osmotic experiments on lipid bilayers and liposomes indicate that NH_3 and HAc can easily cross the membrane as uncharged species, whereas NH_4Cl or NaAc does not cross easily because of the low permeability of the bilayer to Na^+ and to Cl^- (Bangham et al., 1967; Scarpa and DeGier, 1971; Singer and Bangham, 1971). Korenbrot and Cone (1972) have shown that dark-adapted outer segments shocked with NH_4Ac recover at a faster rate than after an equivalent NaCl shock, and that this recovery is not affected by flash illumination before the osmotic shock. This behavior shows that light does not control the influx of NH_4Ac.

In addition, we find that in the dark or after a bleach, outer segments shocked with NH_4Cl also recovered at a faster rate than after an equivalent NaCl shock. In three experiments, outer segments shocked in the dark had a volume of $89\pm2\%$ of pre-shock volume at 2.9 s after shock and recovered to $99\pm1\%$ at 6.4 s. Similarly, in one experiment bleached outer segments had a volume of 86% at 2.9 s and recovered to 98% at 6.4 s. Thus, unlike a NaCl shock (see Fig. 1 legend), after an equivalent (3.5 Is) NH_4Cl shock there is significant volume recovery before the 2.9-s observation and essentially complete recovery at 6.4 s. These results indicate that light does not control the entry of Cl^-, and inasmuch as NH_4Cl entered the outer segment more rapidly than NaCl, this behavior also shows that Na^+ entry, and not Cl^- entry, is rate-limiting after a NaCl shock.

Dark-adapted outer segments shocked with NaAc recovered at a rate similar to that in NaCl, and this recovery was inhibited by light. This again shows that light regulates the influx of Na^+. Taken together, these experiments indicate that light controls the influx of Na^+, not Cl^-, and that in the dark the rate of volume recovery after a NaC shock is limited by the Na^+ permeability.

Osmotic Techniques

The osmotic responses of outer segments and outer segment fragments were studied with a variety of techniques. Preliminary experiments with the glass slide technique and the Nucleopore filter technique indicated that there is greatly diminished volume recovery, if any, after a hyperosmotic NaCl shock using these techniques. Other laboratories have also observed comparable results using techniques similar to the glass slide technique (Cobbs and Hagins, 1974; Bownds and Brodie, 1975) and using X-ray diffraction (Chabre and Cavaggioni, 1975). These results suggest that the NaCl permeability of the outer segments is markedly reduced in these other techniques. We have attempted to discover the cause of this apparent technique-dependent lability of the outer segment P_{Na}. We find that one important factor appears to be the oxidation of the outer segments. It has been reported that oxidation apparently weakens rhodopsin-lipid interactions in the frog ROS membrane (Novikov et al., 1975) and abolishes the rabbit electroretinogram (ERG) a- and b-waves (Hiramitsu et al., 1975). The time-dependent increase in fragility and loss of membrane integrity of ROS membranes can be slowed by using nitrogen or argon atmospheres (Farnsworth and Dratz, 1976). These degradative changes are

apparently due to lipid peroxidation. In addition, Bownds and Brodie (1975) have reported that the dark swelling rate of isolated frog outer segments was increased by bubbling argon through the solution (i.e., by purging O_2 from the solution).

Ethylenediamine tetraacetic acid (EDTA) is an effective inhibitor of lipid peroxidation in a number of in vitro systems (Kohn and Liversedge, 1944; McKnight et al., 1965), and recently Dratz and colleagues[1] (see also Farnsworth and Dratz, 1976) demonstrated that CaEDTA is an effective ROS membrane antioxidant. We have found that outer segments shocked osmotically with NaCl fail to recover in the dark using the modified glass slide technique. This finding is in agreement with the results reported by Cobbs and Hagins (1974). However, we found that when outer segments were shocked in the presence of 3 mM CaEDTA, or when they were shocked under nitrogen atmosphere, the outer segments did recover. Substantial recovery occurred in <3–5 min in the dark; no recovery was seen in outer segments which had been bleached (see Table II).

TABLE II
EFFECT OF ANTIOXIDANTS

	% pre-shock length at 3–5 min after shock	
	Dark	Light
Control	80±5% (6)	79±2% (5)
CaEDTA	96±4% (6)	76±7% (6)
N_2	88±2% (6)*	79±6%(6)*

Results expressed as mean±SD. Numbers in parentheses indicate numbers of experiments.
* Significantly different ($P < 0.01$).

Another factor which may affect the condition of the Na^+ channels is the extent to which the outer segments are diluted with saline solution. For example, shaking the retina into 2 liters as compared with 0.02 ml of saline solution may result in a 20-fold decrease in the rate of volume recovery (Bownds and Brodie, 1975). This "volume effect" may be related to the oxidation effect.

In the continuous flow technique, the retina is gently shaken in the loading well, and then the well is immediately sealed. The outer segments are not exposed again to air atmosphere until after completion of the experiment. All the other techniques involve longer periods of exposure to air. In addition, unlike most of the other techniques, the ROS suspension in the loading well is relatively concentrated, and the ratio of endogenous retina fluid to saline solution is relatively high. Hence, several aspects of the continuous flow technique tend to limit the extent of ROS oxidation and dilution.

In contrast to the other osmotic techniques we investigated, as well as the techniques used by other investigators, the continuous flow technique yields results very similar to those obtained electrophysiologically in the intact retina. The rate of Na^+ entry in the dark determined with the continuous flow technique agrees well with the Na^+ dark current measured electrophysiologically

[1] Dratz, E.A. Personal communication.

in the intact retina (Table III). Also, with this technique the isolated outer segments exhibit essentially the same photosensitivity as found with extracellular recording of the dark current in the intact retina (Hagins et al., 1970; Zuckerman, 1973), and the effects of low Ca^{++} activity in the presence of a Ca^{++} ionophore are comparable (Hagins and Yoshikami, 1974). Therefore, all results reported below are based on the continuous flow technique.

Two important questions concerning this technique have been investigated. Some of the rods fragment even with gentle shaking of the retina, and this raises questions about whether the fragments remain photosensitive. One way of answering these questions is exhibited in Fig. 2. These histograms show the distribution of the lengths of the outer segments and fragments in three experiments where every outer segment and fragment in the microscope field was measured. In the microscope, the depth of focus and the width of the field were great enough to allow measurement of virtually all the outer segments and

TABLE III
DARK Na^+ INFLUX

| | | Determined | |
	Temp	electrophysiologically	osmotically
		Na^+ *per rod/s*	
Rana pipiens	21–23°C		21×10^{8}*
Rana catesbiana	22°C	1.5×10^{8}‡	
Necturus maculosus	~22°C	13×10^{8}§	
Rat	31–33°C	4.4×10^{8}‖	
	21–23°C		0.9×10^{8}*

* Korenbrot and Cone (1972).
‡ Zuckerman (1973).
§ Werblin (1975).
‖ Hagins et al. (1970).

fragments emerging from the exit hole. About 300 outer segments and fragments were measured for each of these histograms.

The uppermost histogram shows the distribution of lengths in standard saline solution. At 2.9 s after a hyperosmotic NaCl shock, outer segments shrank to ≅75% of their initial volume. The outer segments recovered to ≅94% of their initial volume at 9.7 s after the shock. However, outer segments which were bleached before being shocked did not recover in volume, remaining at ≅75% of their initial volume. The dotted-line histograms show the compressed distribution of lengths of unshocked outer segments in the standard saline solution for each experiment. For each pair of distributions, the outer segments that were measured to obtain the data came from the same suspension. Small variations in the shapes of the histogram envelopes for each of the three experiments were due in part to differences in the three separate suspensions from which the outer segments came. For each suspension, the distribution in standard saline (i.e., the control) was linearly compressed along the length axis (without shifting the origins) to make the means of the two histograms coincide. If all the outer segments and fragments are osmotically active, they should all

shrink or recover to the same extent, and thus the linearly-compressed control histogram should coincide with the histogram of the shocked outer segments. Similarly, if all the outer segments and fragments are photosensitive, after a bleach all of them should shrink to the same extent and should remain shrunken. Indeed, in each case, the good match between the pairs of histograms indicates that essentially all the outer segments and fragments are both osmotically active and photosensitive.
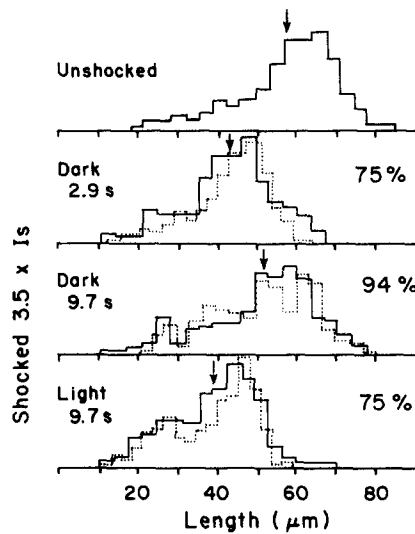

FIGURE 2. Histograms showing the distribution of the lengths of outer segments and outer segment fragments from three experiments. The continuous-line histograms show the distribution of lengths of unshocked outer segments, outer segments 2.9 and 9.7 s after 3.5-Is NaCl shocks in the dark, and outer segments 9.7 s after a 3.5-Is NaCl shock after a bleach. The mean length of outer segments and fragments for each continuous-line histogram is indicated by the arrow. The dotted-line histograms depict the distribution of lengths of the unshocked outer segments and fragments for each experiment: the length scale for the dotted-line histograms has been linearly compressed to make the means of the two distributions coincide. The numbers to the right of the pairs of histograms indicate the mean lengths of the outer segments and fragments after NaCl shocks with respect to the mean lengths of unshocked outer segments and fragments from the same outer segment suspension. Each of the histograms was normalized to the same area.

In addition, the fluorochrome, N,N'-didansyl cystine (DDC), was used to assess the osmotic intactness of the ROS plasma membranes and to determine whether the continuous flow technique induced "leakiness" (assay described by Yoshikami et al., 1974). For each of four experiments, a retina was gently shaken in the loading well of the continuous flow apparatus. An aliquot of the ROS suspension was withdrawn and assayed with 10 μM DDC. The same ROS suspension was then used for an osmotic shock experiment with the continuous flow technique. The syringes were driven at the fastest rate, the rate used for a

2.9-s time point. An aliquot of the ROS suspension which emerged from the exit hole was then assayed. The percentage of DDC-leaky outer segments and fragments in the loading well was 13 ± 6%. For the outer segments and fragments which had experienced the hyperosmotic shock in the continuous flow apparatus, the percentage was unchanged; 13 ± 6% were DDC leaky. Hence, most outer segments and fragments are intact, and the continuous flow technique did not induce leakiness.

H^+ *Dependence*

The effect of protons on the rate of volume recovery after a hyperosmotic NaCl shock in GGP-buffered saline solutions is shown in Fig. 3. The results show that, in the dark, as the external pH decreased, the volume recovery rate also decreased. In other words, as the concentration of H^+ increased, the Na^+ permeability was reduced. For osmotic shocks after a bleach, no detectable recovery in volume occurred at either pH studied.

In the dark, the decrease in recovery rate with decrease in pH was reversible. Two methods were used to check reversibility. One method involved dissecting a retina into pH 5.5 standard saline, letting the retina incubate for ≅4 min in
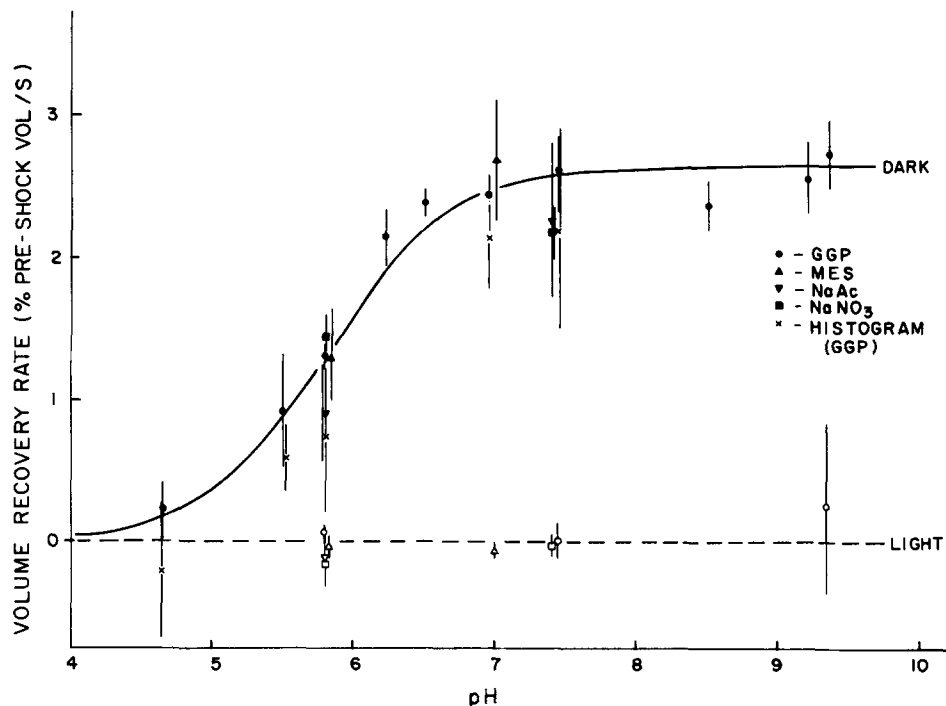
FIGURE 3. Effect of H^+ on volume recovery rates after 3.5-Is NaCl shocks in GGP- and MES-buffered saline solutions in the dark (filled symbols) and after a bleach (open symbols). Data are shown for outer segments shocked in GGP with 3.5 Is NaCl, 3.5 Is $NaNO_3$, and 3.5 Is NaAc. Rates derived from histogram analysis of NaCl in GGP data are also plotted. Error bars represent ±SD.

this solution, then transferring the retina to pH 7.4 standard saline, and proceeding from this point with the usual hyperosmotic NaCl shock experiment. The second method involved dissecting the retina into pH 4.75 saline solution (with 2 mM GGP buffer), letting it incubate for $\cong 4$ min in this solution, then shaking the retina into the loading well filled with the same solution (the syringe connected to the loading well was also filled with the same pH 4.75 solution), and shocking with a solution made hyperosmotic by adding NaCl to a pH 7.7 saline solution (with 20 mM GGP buffer). The measured pH after the mixing of these solutions was 7.4. Thus, outer segments were brought back to normal pH during the shock. In both methods, the rate of volume recovery after a 3.5 Is NaCl shock did not differ significantly from that observed in experiments where outer segments were exposed only to pH 7.4 saline solutions. The second method shows that the effect of lowering the pH can be reversed in less than a few seconds.

The H^+ effect is apparently buffer-independent inasmuch as the effect was the same whether the buffer was made from the mixture of glycylglycine and piperazine or from MES (Fig. 3). Experiments were also performed to determine whether the anion had any effect. As shown in Fig. 3, similar results were obtained in both dark and light in experiments where NO_3^- or Ac^- was substituted for Cl^- in both the standard saline and the hyperosmotic solutions. The results of these control experiments provide further evidence that it is only the Na^+ channel that is being blocked by H^+ (and by light). Finally, to insure that the decrease in recovery rate with decrease in pH was not due to a pH-dependent decrease in restoring force, outer segments were shocked with glycerol in standard saline. The rates of recovery after hyperosmotic glycerol shocks at pH 5.3 and pH 7.4 were essentially the same.

In Fig. 3, the smooth curve drawn through the data points is the theoretical titration curve of a weak acid with a pK_a of 5.8. The apparent pK_a was determined by sliding the theoretical curve along the pH axis to make the best fit by eye. The good fit of this curve to the data suggests that the permeability of the Na^+ channel is blocked when an acidic group associated with it binds a proton.

Ca^{++} Dependence

In addition to protons, Ca^{++} ions also decrease the permeability of the Na^+ channel. Fig. 4 A shows the effect of external Ca^{++} activity on the rate of recovery after hyperosmotic NaCl shock. At the normal level of external Ca^{++} (2 mM), the outer segments recovered in the dark, but not after a bleach. Lowering the external Ca^{++} activity below 2 mM, had little effect, i.e., there was still a NaCl influx in the dark, but not in the light. Thus, low external Ca^{++} has little effect on the Na channel. On the other hand, Korenbrot and Cone (1972) showed that, if the external Ca^{++} activity was increased to 10 mM, the NaCl influx was blocked both in the dark and in the light. In a similar manner, 20 mM Ca^{++} was found by Yoshikami and Hagins (1973) to suppress the dark current in the intact rat retina.

To investigate the effects of cytoplasmic Ca^{++}, the ionophores X537A (lasalocid) and A23187 were used. X537A is a monocarboxylic acid antibiotic which can

transfer both univalent and divalent cations across lipid bilayers and cell membranes (see e.g., Scarpa et al., 1972; Célis et al., 1974). X537A can also bind and transport the Tris buffer cation and organic amines. The mechanism by which X537A increases the Ca^{++} permeability of membranes involves the
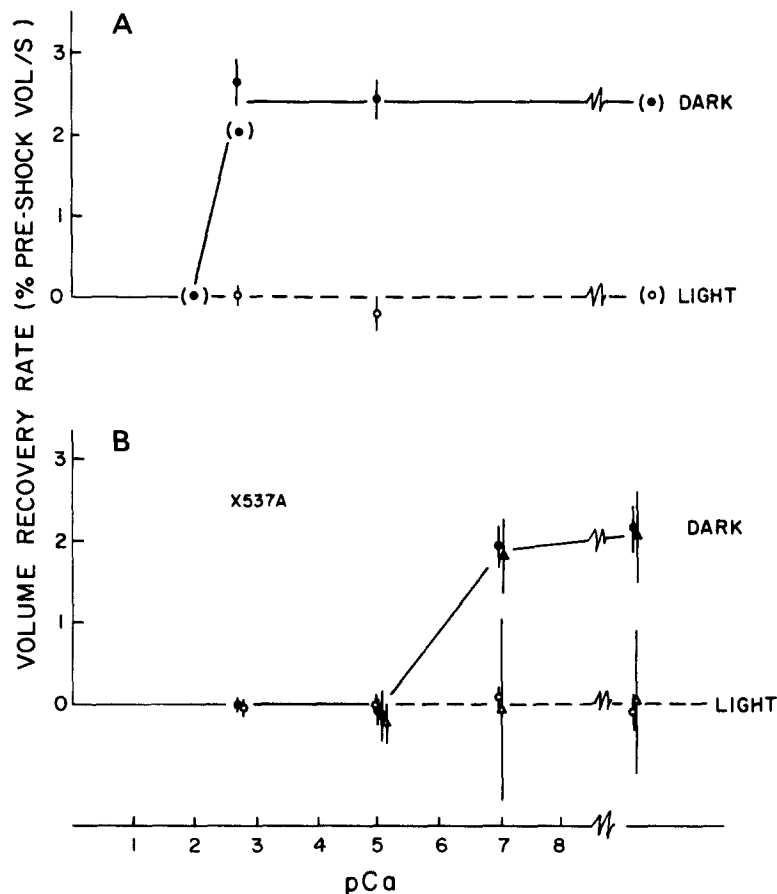

FIGURE 4. Effect of Ca^{++} on the recovery rate after a 3.5-Is NaCl shock in the dark (●) or after a bleach (○). (A) shows the rates of recovery in standard saline vs. the Ca^{++} activity in the saline solutions. The data points in parentheses ( ) are taken from Korenbrot and Cone (1972). (B) shows the rates of recovery in saline solutions containing 10 μM X537A. The data points at the far right of the graphs were obtained at pCa 9–10. Data points derived from histograms (triangles) are also plotted. Error bars represent ±SD.

formation of a lipophilic complex in which two ionophore molecules are needed to solubilize the Ca^{++} ion in the hydrocarbon region of the membrane.

Like X537A, A23187 is also a monocarboxylic acid antibiotic that forms neutral 2:1 complexes with divalent cations (see e.g., Smith and Duax, 1976). A23187 can also transfer divalent cations across lipid bilayers and biological membranes (see e.g., Scarpa et al., 1972; Kafka and Holz, 1975), including liposomes

prepared from lipids extracted from retinas (Hyono et al., 1975) and bovine outer segments (Bonting and Daemen, 1976). But unlike X537A, it apparently does not act as a monovalent cation ionophore because its affinity for monovalent cations is very small (see e.g., Pfeiffer et al., 1974).

Using X537A or A23187, it may be possible to change intracellular Ca^{++} levels and observe the consequent effect of Na^+ channel permeability. Indeed, Hagins and Yoshikami (1974), using radioactive Ca^{++}, have shown that X537A causes a large efflux of Ca^{++} from frog outer segments when external Ca^{++} activity is low. Bonting and Daemen (1976) have shown that A23187 produces a similar effect on bovine outer segments. A23187 and X537A have also been shown to release Ca^{++} from sonicated bovine ROS discs (Smith et al., 1977). X537A also enhances Ca^{++} leakage from liposomes prepared from lipids extracted from bovine retinas (Hyono et al., 1975).

Fig. 4 B shows the recovery rate after hyperosmotic NaCl shock in the presence of X537A plotted vs. the extracellular pCa. The pCa is defined as $-\log(Ca^{++}$ ion activity). In contrast to the results obtained without the ionophore, at the normal level of Ca^{++} (2 mM) there was no detectable NaCl influx in the dark. In fact, with X537A present, no volume recovery was observed except with solutions in which the external Ca^{++} activity was lower than 10^{-5} M. Thus, these observations suggest that cytoplasmic Ca^{++} blocks the Na^+ channel much more effectively than extracellular Ca^{++} does. In the presence of X537A, no recovery was observed at any Ca^{++} activity when the ROS suspension was bleached before the hyperosmotic NaCl shock. Light was effective in inhibiting volume recovery even when the retina was exposed to X537A for up to 13 min. These effects on Na^+ permeability of low Ca^{++} activity in the presence of X537A are in close agreement with their effects on the dark current observed with extracellular electrodes in the isolated rat retina (Hagins and Yoshikami, 1974).

A few experiments were performed in the presence of 1 μM A23187. With 10^{-5} M Ca^{++} outside, no volume recovery occurred in the dark or in the light. When the activity of Ca^{++} was further decreased by using 0.5 mM EGTA and no added Ca^{++}, significant recovery occurred in the dark but not in the light. Hence, similar results were obtained in the presence of either X537A or A23187.

DISCUSSION

Osmotic Techniques

The validity of using osmotic techniques to study the permeability characteristics of the plasma membrane of the outer segment has been checked in a number of ways. Korenbrot et al. (1973) have shown that the light-sensitive Na^+ influx observed in these experiments must occur through the plasma membrane. In addition, the histograms of Fig. 2 indicate that, in the continuous flow technique, essentially all of the outer segments and fragments are both osmotically active and light-sensitive. The DDC assay also shows that most isolated outer segments, as well as fragments, are impermeant to this dye both before and after the shock in the continuous flow technique.

In addition to the continuous flow technique, a variety of other osmotic techniques have been investigated. Most techniques (e.g., the Nucleopore filter

and glass slide techniques) yield results which suggest that the light-regulated Na^+ channel is highly labile, i.e., little or no recovery in volume occurs after hyperosmotic NaCl shocks in the dark.

Using techniques which allowed the osmotic behavior of single outer segments to be followed, Cobbs and Hagins (in a preliminary report in 1974) concluded that under their conditions outer segments do not recover in volume by even 2% during the first 20 min after a hyperosmotic NaCl shock. However, using a glass slide technique, Bownds and Brodie (1975) found significantly different results: the outer segments did recover in volume, and they recovered in volume at a rate of $\cong 2\%$ of their pre-shock volume per minute. Thus, they recover at least 20 times more rapidly than reported by Cobbs and Hagins. Using another osmotic technique involving a Coulter Counter, Bownds and Brodie found a dark recovery rate of 0.5–1.0%/min. Although the volume recovery rate with these techniques is much slower than with the continuous flow technique, it is important to note that the rate of volume recovery is still highly sensitive to light (Bownds and Brodie, 1975).

It appears that oxidation of the membrane may be a key factor in explaining the differences in techniques. Our experiments show that, in the presence of CaEDTA or under a nitrogen atmosphere, the dark recovery rate after a hyperosmotic NaCl shock using the glass slide technique is greatly increased. In addition to oxidation, or perhaps because of oxidation, dilution and aging can decrease the recovery rate (Bownds and Brodie, 1975). In contrast to the continuous flow technique, many of the other techniques involve dilution of the outer segments into a large volume of saline solution. Moreover, the suspension of outer segments in the continuous flow technique is immediately sealed after shaking the retina, whereas all the other techniques involve longer periods of exposure to air.

The results obtained using the continuous flow osmotic technique, in every case where a direct comparison can be made, agree reasonably with results observed in the intact retina using electrophysiological techniques. For example, the photosensitivity of the receptors is found to be very similar with both the osmotic and the electrophysiological techniques (see Korenbrot and Cone, 1972). Also, the osmotically determined rate of Na^+ entry in the dark agrees well with the dark current determined electrophysiologically in the intact retina (Table II), and the osmotically determined light dependence of the Na^+ resistance (Korenbrot and Cone, 1972) is fit by the same function that Baylor and Fuortes (1970) inferred from intracellularly recorded data. Finally, the results obtained in the present investigation (with and without X537A) are similar to the results obtained by Hagins and Yoshikami (1974) using extracellular electrodes in the isolated rat retina. Thus, there is good reason to believe that the characteristics of the light-regulated Na^+ channel, as observed under the conditions of our continuous flow experiments, are very similar to the in vivo characteristics.

H^+ Dependence

It has been known for some time that absorption of a photon by a vertebrate rhodopsin molecule results in the net uptake of one proton during the early

stages of the bleaching sequence. Changes in pH in response to light have been measured in suspensions of outer segments and rhodopsin solutions (see e.g., Radding and Wald, 1956; Falk and Fatt, 1966; Ostroy, 1974). It has also been found that bacteriorhodopsin, a rhodopsin-like protein from a halophilic bacterium (*Halobacterium halobium*), appears to be a light-driven proton pump (see e.g., Oesterhelt and Stoeckenius, 1973). Because of these observations, it is of interest to investigate the effects of protons on the permeability of the Na^+ channel in the vertebrate photoreceptor.

The present investigation is the first to examine directly the H^+ dependence of the Na^+ channel permeability in a vertebrate photoreceptor. The results of previous investigations using extracellular recording techniques (Sillman et al., 1972; Ward and Ostroy, 1972; Gedney and Ostroy, 1974) are difficult to interpret in that it was impossible to determine the site(s) of action of the pH effects. However, Bownds and Brodie (1975) found that, at pH 5.5, the "dark swelling" of isolated outer segments is reduced to the same extent as after illumination.

The present study has shown that the Na^+ channel in the plasma membrane of the ROS is reversibly blocked by lowering the external pH, and that this pH dependence follows the theoretical titration curve of a single acidic group with an apparent pK_a of 5.8. This behavior suggests that the Na^+ channel is blocked when a high field strength acidic group binds a proton.

The H^+ dependence and cation selectivity of the Na^+ channels in nerve and in muscle suggest that a high field strength anionic group forms part of the "selectivity filter" which gives the Na^+ channel its high ionic specificity (see e.g., Hille, 1975). The similarity between the Na^+ channels in the ROS, nerve, and muscle suggests that the light-regulated Na^+ channel may also possess a high field strength anionic site as part of its selectivity filter.

We do not know from which side of the plasma membrane H^+ can approach this anionic group in that our experiments with NaAc indicate that, in the dark, the outer segment plasma membrane may be highly permeable to H^+. Unlike outer segments, liposomes and bacteria do not recover in volume or swell after exposure to NaAc. However, volume recovery or swelling do occur when either a protonophore or an agent which allows a Na^+-H^+ exchange is added. For example, NaAc enters if nigericin is added to liposomes (Singer and Bangham, 1971) or if monensin is added to bacteria (Visser and Postma, 1973). Moreover, in liposomes made permeable to K^+ (by addition of valinomycin), KAc does not enter unless the protonophore, salicylate, is added (increasing the permeability to H^+) (Singer, 1973). In osmotic experiments with mitochondria, Mitchell and Moyle (1969) concluded that NaAc can enter the mitochondria inasmuch as Na^+ exchanges with H^+ across the membranes. Therefore, because NaAc rapidly enters the outer segments in our experiments, it appears the plasma membrane not only needs to be permeable to Na^+, but must also permit H^+ to pass through the membrane either through a channel or by an exchange with Na^+ (alternatively, OH^- could pass through a channel or be co-transported with Na^+). In view of the Eisenman theory of equilibrium selectivity (see e.g. Eisenman, 1961), it would not be surprising if the Na^+ channels were themselves permeable to

H^+. Indeed, Eisenman's theory predicts that a high field strength anionic site would make the channel more highly selective for H^+ than for Na^+.

Ca^{++} Dependence

The hypothesis that Ca^{++} is the internal transmitter in vertebrate rods and cones has received suggestive support in several recent studies. It has been demonstrated that changes in external Ca^{++} affect (a) the dark and photo-currents of rods in the isolated rat retina (Hagins et al., 1970; Yoshikami and Hagins, 1970, 1971, 1973, 1975), (b) the dark influx of Na^+ ions through the Na^+ channel in isolated frog ROS (Korenbrot and Cone, 1972), (c) the receptor-related components of the electroretinogram of the isolated frog retina (Pautler and Su, 1971; Snyder, 1974) and rat retina (see e.g. Winkler, 1974), and (d) the intracellularly recorded membrane potentials (in dark and light) of toad photoreceptors (Brown and Pinto, 1974), carp cones (Kaneko and Shimazaki, 1975) and turtle cones (Cervetto and Piccolino, 1974). In general, raising the external Ca^{++} activity decreases the amplitude of the light-response, whereas lowering the external Ca^{++} activity increases the amplitude. External Ca^{++} also mimics some of the effects of steady background light. In a recent preliminary report, Hagins and Yoshikami (1977) found that the addition of Ca^{++} buffers to the photoreceptor cytoplasm decreased the amplitude of the extracellularly recorded responses of rat rods to dim light flashes and did not affect the responses to bright flashes.

Several studies also report that there is a light-induced release of Ca^{++} from discs and (or) outer segments (Abrahamson, et al., 1974; Baker and Mason, 1974; Hendriks et al., 1974; Liebman, 1974, 1976; Hemminki, 1975; Weller et al., 1975; Bonting and Daemen, 1976; Shevchenko, 1976) and from vesicles prepared by sonication of reconstituted rhodopsin-phospholipid or ROS membrane suspensions[2] (Mason et al., 1974; Smith et al., 1977). Some reports indicate no measurable light-induced release of Ca^{++} from discs (Sorbi and Cavaggioni, 1975; Szuts and Cone, 1977).

The use of antibiotic ionophores has greatly aided investigations of the effects of intracellular ions. Using extracellular recording techniques on the isolated rat retina, Hagins and Yoshikami (1974) examined the effects of varying external Ca^{++} activity in the presence of the ionophore X537A. With X537A present, they found that about 2,000 times less Ca^{++} was needed to suppress the dark current than in untreated retinas.

The present experiments are consistent with the hypothesis suggested by the electrophysiological results of Hagins and Yoshikami (1974) that Ca^{++} suppresses the dark current by blocking the Na^+ channels in the plasma membrane of the ROS. Without the ionophore X537A present, Korenbrot and Cone (1972) found that, in the dark, the permeability of the Na^+ channel in the ROS was markedly reduced when external Ca^{++} activity was raised from 2×10^{-3} M to 10^{-2} M. On the other hand, with X537A present, we find that raising Ca^{++} from 10^{-7} M to only 10^{-5} M is sufficient to block the Na^+ channels. Hence, cytoplasmic Ca^{++} appears to reduce Na^+ permeability much more effectively than external Ca^{++}.

[2] Hong, K. and W. L. Hubbell. Personal communication.

These observations indicate that the electrophysiological effects of intracellular Ca^{++} on the dark current seen by Hagins and Yoshikami are indeed due to effects of Ca^{++} on the Na^+ channels in the plasma membrane of the outer segment and not to effects elsewhere in the visual receptor. The ability of X537A to transfer Ca^{++} across the outer segment plasma membrane has been verified by preliminary experiments which have shown that exposure of isolated frog outer segments to X537A results in a large efflux of Ca^{++} (Hagins and Yoshikami, 1974).

The observation that light can still inhibit volume recovery in the presence of either X537A or A23187 is interesting and unexplained. If Ca^{++} is the transmitter and if the ionophores increase the Ca^{++} permeability of the plasma and disc membranes, it would be expected that the effect of light would disappear in solutions with very low Ca^{++} activity. But the effect of light persisted even when the retinas were exposed for up to 13 min to either of the ionophores. This appears to contradict the Ca^{++} hypothesis. However, it may imply only that the ionophores did not induce sufficient Ca^{++} permeability in the ROS membranes to overcome light-regulated release of Ca^{++}.

Comparison with Other Na^+ Channels

The characteristics of the electrically-excited Na^+ channels in both nerve and muscle are quite similar to those we have observed in the light-regulated Na^+ channels in the ROS. For example, all three channels are highly selective since they are much more permeable to Na^+ than to K^+.

The H^+ dependence of the Na^+ channels in nerve (see e.g., Hille, 1968; Woodhull, 1973) and in muscle (Campbell and Hille, 1976) has been studied by a number of investigators and is remarkably similar to that in the ROS. These investigators have shown that the permeability of the Na^+ channels in frog myelinated axons and frog skeletal muscle fibers decreases with decreasing pH. Their data fit the titration curve of a single acidic group with an apparent pK_a of 5.4 at zero membrane potential for nerve (see Woodhull, 1973) and an apparent pK_a of 5.3 at zero membrane potential for muscle (Campbell and Hille, 1976). Our data also fit the titration curve of a single acidic group with the slightly higher apparent pK_a of 5.8.

Moreover, the Na^+ channels in nerve, muscle, and photoreceptor are also blocked by Ca^{++} ions. External Ca^{++} ions at high concentrations block Na^+ channels in frog nodes with an apparent K_{diss} of 23 mM (Woodhull, 1973) and in frog muscle fibers with an apparent K_{diss} of 80 mM (Campbell and Hille, 1976). The light-regulated Na^+ channel is similarly blocked by external Ca^{++} ions. Raising the external Ca^{++} concentration from 2 mM to 10 mM blocks Na^+ channels in frog outer segments (Korenbrot and Cone, 1972), and raising the Ca^{++} concentration to 20 mM suppresses the Na^+ dark current in rat rods (Yoshikami and Hagins, 1973).

Two significant differences between the Na^+ channels in the axon and in the vertebrate photoreceptor should be mentioned. One difference is that photoreceptor Na^+ channels are not sensitive to tetrodotoxin (TTX). TTX blocks voltage-regulated Na^+ channels from the outside, but not from the inside (see e.g., Narahashi et al., 1966). In contrast, light-regulated Na^+ channels appear to

be insensitive to TTX since the PIII component of the electroretinogram is not significantly affected by externally applied TTX (see e.g. Murakami and Shigematsu, 1970).

Another difference is that the Na^+ channels in the photoreceptor are much more sensitive to cytoplasmic Ca^{++} than are those in the axon. Apparently, internal Ca^{++} at concentrations up to 10 mM has little, if any, effect on ionic currents in squid axon (Begenisich and Lynch, 1974). On the other hand, in the presence of Ca^{++} ionophores, 10 μM external Ca^{++} is sufficient to block Na^+ permeability in outer segments. The differences in Ca^{++} sensitivity and in TTX sensitivity may be due in part to differences in accessibility of the high field strength anionic site associated with the Na^+ channel.

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