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Light-dependent Ion Influx into Toad Photoreceptors

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ABSTRACT To measure the influx of Na^+ and other ions through the light-dependent permeability of photoreceptors, we superfused the isolated retina of the toad, *Bufo marinus*, with a low- Ca^{2+} (10^{-8} M), low- Cl^- Ringer's solution containing 0.5 mM ouabain. Under these conditions, the membrane potential of the rod is near zero and there is no light-induced potential change either in the rod or in more proximal neurons. The photoreceptors, however, continue to show a light-dependent increase in membrane resistance, which indicates that the light-sensitive channels still close with illumination. Dark-adapted retinas show a larger $^{22}\text{Na}^+$ accumulation than do light-adapted retinas. The extra accumulation of $^{22}\text{Na}^+$ into dark-adapted retinas can be removed if the retinas are washed in darkness with low- Ca^{2+} Ringer's solutions, or if the ionophore gramicidin D is present in the perfusate. The additional accumulation in dark retinas corresponds to a flux of at least 10^9 Na^+ per receptor per second, which is close to the value of the photoreceptor dark current. The light-dependent uptake of $^{22}\text{Na}^+$ can be prevented by exposing the retinas to Ca^{2+} during the incubation period, but is restored if the phosphodiesterase inhibitor IBMX is added to the perfusate. A significant light-dependent ion accumulation can be observed for the cations K^+ , Rb^+ , Cs^+ , and Tl^+ , in addition to Na^+ , but not for methylamine, choline, or tetraethylammonium.

INTRODUCTION

Vertebrate photoreceptors contain an ion conductance mechanism whose gating is controlled by chemical events that are modulated by photon absorption (see Hubbell and Bownds, 1979; Fain and Lisman, 1981). In darkness the light-sensitive channel¹ has a relatively high sodium permeability, and sodium enters the cell down its electrochemical gradient. Illumination leads to a decrease in permeability and a hyperpolarization of the membrane.

In this report we present the first demonstration that the permeability of

¹ Although we use the word "channel" to describe the permeability mechanism, the physical nature of the mechanism, whether it is a pore or carrier, has not yet been established.

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the photoreceptor light-dependent channel can be monitored using radiotracer flux measurements in intact retinas. Several studies have demonstrated that radioactive tracer fluxes in whole retinas (Sorbi and Cavaggioni, 1971; Cavaggioni et al., 1972) or in isolated photoreceptor outer segments (Cavaggioni et al., 1973) can be influenced by illumination; however, in these previous studies the light-dependent ion movements could not be unambiguously attributed to the light-sensitive permeability. For example, the fluxes previously measured could have been caused by voltage-sensitive channels (see Fain and Lisman, 1981) or to postsynaptic neurons whose permeabilities are also affected by illumination. In our experiments we eliminated changes in voltage and synaptic transmitter release that normally occur with illumination by incubating retinas in low- Ca^{2+} solutions that contained the cardiac glycoside ouabain. We shall demonstrate that in this solution the rods show no voltage responses, but the light-dependent channels still close when the photoreceptor is exposed to light. We shall also give evidence for an influx and efflux of sodium ions in the retinas that is inhibited by illumination.

Some of the experiments in the present report have been presented at the meetings of the Biophysical Society (Woodruff and Fain, 1980) and the Association for Research in Vision and Ophthalmology (Fain and Woodruff, 1981).

METHODS

Materials and Solutions

The experimental animals were large female toads, *Bufo marinus*, obtained from Charles Sullivan (Nashville, TN). They were kept on a 12 h dark/12 h light cycle for at least 2 wk before using them in experiments. We fed them live mealworms three times each week. Before each experiment the toads were dark-adapted for ~18 h.

The principal solutions used are listed in Table I. Solution A is normal toad Ringer's solution (modified from Brown and Pinto, 1974). Solutions B–D are low- Cl^- Ringer's solutions with, respectively, normal Ca^{2+} , low Ca^{2+} , and low Ca^{2+} plus 0.5 mM ouabain. The Cl^- was substituted with CH_3SO_3^- in some experiments to prevent photoreceptor swelling in low Ca^{2+} (Bastian and Fain, 1982). The free- Ca^{2+} concentration in solutions C and D was calculated to be 1.0×10^{-8} M (Caldwell, 1970). Solutions E, F, and G are high- K^+ , low- Na^+ solutions analogous to solutions B, C, and D, respectively. Solution H is a simple high- Ca^{2+} salt solution that we used to wash the retinas after exposing them to radioactivity (see below). The solutions were continuously bubbled with 100% oxygen at room temperature during an experiment.

Ouabain, IBMX (isobutylmethylxanthine), EGTA, gramicidin D, and the components of our Ringer's solutions listed in Table I were purchased from Sigma Chemical Co., St. Louis, MO. $^{22}\text{NaCl}$, ^{42}KCl , $^{86}\text{RbCl}$, $^{131}\text{CsCl}$, $^{204}\text{TlNO}_3$, [^3H]choline Cl, and [^{14}C]tetramethylammonium Br were obtained from New England Nuclear, Boston, MA. [^{14}C]methylamine was obtained from Amersham Corp., Arlington Heights, IL.

Electrophysiology and Photostimulation

A detailed description of the procedures and apparatus for making intracellular recordings from rods in the isolated, superfused toad retina can be found in Bastian

and Fain (1979). Micropipettes for intracellular recording were filled with 2 M K acetate and had resistances in normal toad Ringer's solution (solution A) between 100 and 300 M Ω . Retinas were illuminated with full-field, 501-nm light whose unattenuated irradiance was 13.1 log quanta cm⁻² s⁻¹. Light intensity was determined using a calibrated photodiode. Assuming a collecting area of 29.5 μ m (Fain, 1976), we estimate that this unattenuated light bleached 3.9×10^6 rhodopsin molecules in each rod photoreceptor per second. We also determined the rate of photopigment bleaching for the same light by difference spectroscopy using a homogeneous suspension of isolated rod outer segments (Bownds et al., 1971). Approximately 4×10^6 rhodopsin molecules were bleached per rod outer segment per second, a value in good agreement with our estimate based on the collecting area of the rod. In addition to the 501-nm filtering, we used two heat filters in the light beam (KG 3; Melles Griot, Irvine, CA). In the electrophysiological experiment of Fig. 2, we reduced the stimulus intensity with calibrated neutral density filters (Fish-Schurman Corp., New Rochelle, NY).

TABLE I
COMPOSITION OF SOLUTIONS

Solu- tion	NaCl	KCl	CaCl ₂	MgCl ₂	NaHCO ₃	Na ₂ SO ₄	MgSO ₄	NaCH ₃ SO ₃	KCH ₃ SO ₃	EGTA	Oua- bain	Free [Ca ²⁺] _o
	<i>mM</i>											
A	106	2.5	1.8	1.2	0.13	1.8	—	—	—	—	—	1.8×10^{-3}
B	—	—	1.8	—	0.13	1.8	1.2	106	2.5	—	—	1.8×10^{-3}
C	—	—	1.8	—	0.13	1.8	1.2	99	2.5	3.18	—	1.0×10^{-8}
D	—	—	1.8	—	0.13	1.8	1.2	99	2.5	3.18	0.5	1.0×10^{-8}
E	—	—	1.8	—	0.13	1.8	1.2	28	90.5	—	—	1.8×10^{-3}
F	—	—	1.8	—	0.13	1.8	1.2	28	90.5	3.18	—	1.0×10^{-8}
G	—	—	1.8	—	0.13	1.8	1.2	28	90.5	3.18	0.5	1.0×10^{-8}
H	106	2.5	20	—	—	—	—	—	—	—	—	2.0×10^{-2}

All solutions contained 3 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid) and 5.6 mM glucose and were buffered to pH 7.8 by adding NaOH. Solutions containing EGTA took relatively more NaOH to bring the pH to 7.8. We reduced the NaCH₃SO₃ concentration in these cases to maintain the Na⁺ concentration at ~110 mM. Solution osmolarities were measured with a vapor pressure osmometer (Westcor, Inc., Logan, UT) to be between 210 and 225 mosmol.

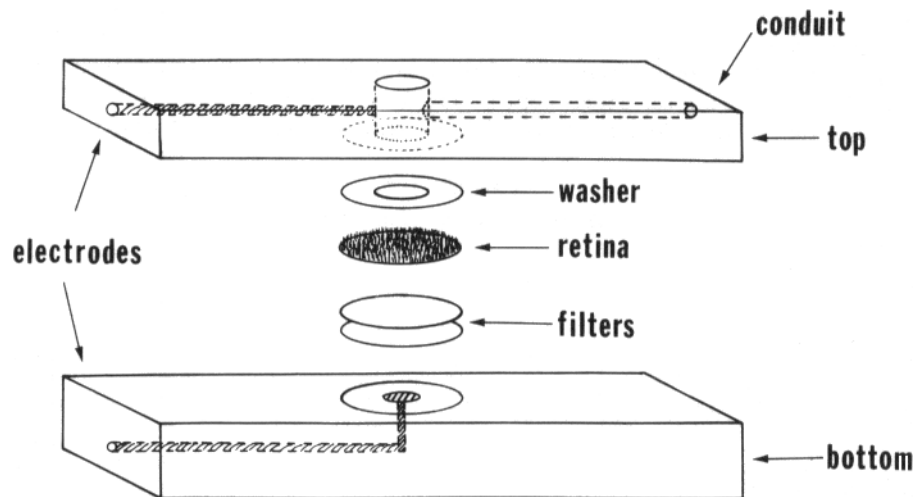
Tracer Flux Measurements

In all experiments, tracer accumulation was estimated by comparing the average radioactive uptake in fully dark-adapted retinas with the average uptake in retinas exposed to the unattenuated light just described. In a typical experiment, we dissected a dark-adapted retina under infrared illumination by removing a small, roughly circular section 7–10 mm in diameter from the pigment epithelium and adsorbed it receptor side up onto a Millipore filter (type HAWP-0013, 0.45 μ m; Millipore Corp., Boston, MA). We used an infrared image converter (Find-R-Scope; F. J. W. Industries, Mt. Prospect, IL) to visualize the dissection and all other experimental manipulations in darkness. We then placed the retina with its filter on top of another filter of the same type, and the retinas with filters were placed into the incubation chamber shown in Fig. 1. The second filter was used as a spacer so that the margin of the retina was held firmly against the teflon washer when the chamber was assembled.

This chamber was similar to the one described by Bastian and Fain (1979). It contained a single inflow/outflow conduit and Ag/AgCl electrodes for monitoring light-evoked transretinal voltages (electroretinogram, ERG). The incubation well volume was ~50 μ l. Solutions were added and removed through the conduit by pipetting (Pipetman, with disposable tips; Rainin Instruments, Brighton, MA).

Throughout the course of an experiment, we removed the solution in the incubation chamber and added a freshly oxygenated solution every 1–2 min to maintain the oxygen tension.

After we placed the retina in the chamber, we incubated it in normal toad Ringer's solution for 8–10 min before exposing it to the various modified Ringer's solutions of Table I. The order of solution incubations and the times of incubation are given in the Results and the figure legends. To introduce radioactivity, we removed the



INCUBATION CHAMBER

FIGURE 1. A three-dimensional view of the two-piece plexiglass perfusion chamber. The retina section was adsorbed to a Millipore filter (13 mm in diameter) by capillarity. A second filter placed under the retina filter served as a spacer, so that the retina was held firmly against the teflon washer when the chamber was assembled. A thin layer of petroleum jelly spread between the chamber halves and on the washer prevented leakage of fluid. A silver-silver chloride electrode countersunk into the middle of the chamber bottom served as the active electrode for recording the ERG. A second electrode placed in the well flush with the wall served as ground for the ERG and intracellular recording.

nonradioactive solution from the incubation well and then added freshly oxygenated solution of the same type containing a small amount of radioactive tracer. When a solution was removed from the chamber, a small amount of it, $\sim 6.5 \mu\text{l}$, remained with the retina. We mixed the radioactive solution with the residual nonradioactive solution by drawing the solution in and out of the chamber a few times. For retinas that were exposed to illumination, we turned on the unattenuated light 10 s before adding the radioactivity. We terminated tracer incubation by removing the radioactive solution and adding nonradioactive Ringer's solution. The radioactive incubation solution was saved after removing it from the chamber so we could determine the specific amount of radioactivity exposed to the retina. We washed the retina six times

with nonradioactive solution (50 μ l each) and then, after the final wash was removed from the chamber, we quickly froze the retina by using Freon-22 (-70°C). The six rinses took ~ 3 min and the final wash contained $\sim 0.01\%$ of the radioactivity of the initial incubation solution. The residual wash left with the retina was only 1–5% of the total accumulated activity. The final wash activity was determined in each experiment, and the amount of tracer in the wash residual was subtracted from the total before calculating Na^+ accumulation. The times of incubation with radioactive tracers and the amounts of radioactivity are given for each experiment in the figure legends.

We removed the frozen retina from the incubation chamber (at -20°C) and placed the circular middle section that was exposed to radioactivity in 10% trichloroacetic acid (TCA) at room temperature. We then centrifuged the TCA-treated samples at 2,200 g for 30 min and took aliquots from the supernatant for liquid scintillation counting, adding 10 ml of Aquasol-2 (New England Nuclear) to each sample. We dissolved the precipitate in 1 M NaOH and determined its protein content by the Lowry method (Lowry et al., 1951). For one of the cations, thallium, we examined the relation between uptake and bath concentration and found that uptake was linear with concentration (coefficient of correlation = 0.98).

RESULTS

To study the permeability of the light-dependent channel, we attempted to measure a light-dependent accumulation of radioactive ions into intact isolated retinas of the toad, *Bufo marinus*, expecting that uptake would be greater (more rapid) in completely dark-adapted retinas, when the photoreceptor light-dependent channels are open, than in light-exposed retinas, when these channels are closed. To minimize any contributions to the light-dependent accumulation that might be made by retinal permeabilities other than the photoreceptor light-dependent channels, we incubated the retinas in low- Ca^{2+} -ouabain Ringer's solution (solution D). Fig. 2 shows the effects of this solution on the intracellular responses of toad rods. The hyperpolarizing response to a bright flash in normal toad Ringer's solution is given in Fig. 2*a*. The resting potential of the cell was about -35 mV, and a 100-ms exposure to 501-nm light that bleached $\sim 2.4 \times 10^5$ rhodopsin molecules ($10.8 \log$ quanta cm^{-2}) hyperpolarized the membrane potential to about -55 mV. After switching to solution C containing 10^{-8} M Ca^{2+} , the resting potential of the cell depolarized to near -5 mV, probably because of a large increase in the light-dependent conductance of the receptor (see, for example, Yau et al., 1981). Exposing the cell in low Ca^{2+} to the same light as in *a* hyperpolarized the cell again to near -55 mV. Large amplitude responses like the one shown in Fig. 2*b* can be obtained for >1 h in 10^{-8} M Ca^{2+} (data not shown); however, the addition of 0.5 mM ouabain rapidly abolishes the light response (Fig. 1*c–g*). The ERG disappears concomitantly. Ouabain has this effect, probably because it inhibits the photoreceptor Na^+, K^+ -ATPase (Frank and Goldsmith, 1972). Because photoreceptors are small and permeable to Na^+ and K^+ in the dark (see Fain and Lisman, 1981), the inhibition of ATPase would be expected to lead to a rapid equilibration of Na^+ and K^+ across the plasma membrane. With the Na^+ and K^+ gradients dissipated, there would be no driving force for Na^+ and K^+ and thus no voltage change when the light-dependent channels closed.

Although there is no voltage response in ouabain, light increased the input resistance of the receptor, which showed that the light-dependent conductance is still capable of closing when the photoreceptor is stimulated. To monitor the input resistance of the rod, 0.1 nA of current was injected through the recording pipette in 250-ms pulses. Fig. 1g shows that 4 min and 12 s after the ouabain addition, a bright flash of light produced an increase in the input resistance. The stable baseline in this trace indicates that the resistance increase was not accompanied by a change in membrane voltage.

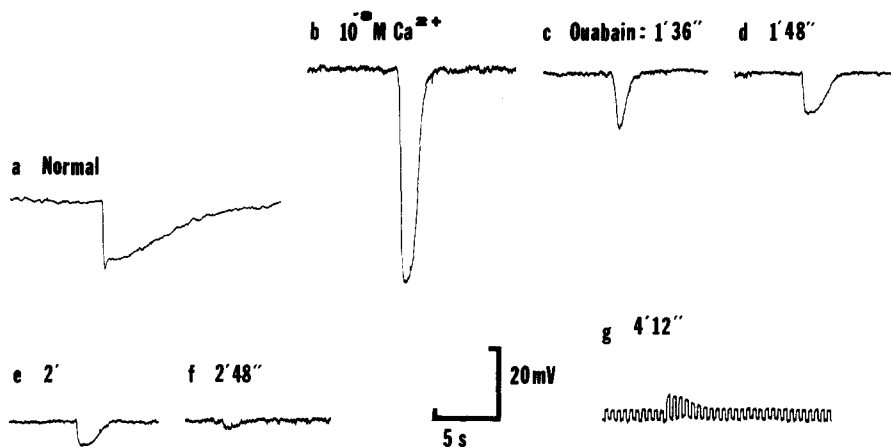


FIGURE 2. The effect of low Ca^{2+} and ouabain on toad rod responses. All responses are to a 100-ms, 501-nm, full-field light with an intensity of $10.8 \log \text{ quanta cm}^{-2}$. (a) The hyperpolarizing response in normal toad Ringer's solution (solution A of Table I). (b) Response after few minutes in low- Ca^{2+} Ringer's solution (solution C). (c-g) Responses between 1 min, 36 s and 4 min, 12 s of switching to low- Ca^{2+} -ouabain Ringer's solution (solution D). In the last trace (g), 0.1-nA current pulses were injected into the recording pipette using a standard bridge circuit. Illumination unbalanced the bridge, which indicates an increase in input resistance of $\sim 33 \text{ M}\Omega$. Note the baseline from which the pulses merged was stable during the light exposure, showing that the light had no effect on membrane potential. Traces in g were retouched to remove stimulus artifacts originating from the current-clamp bridge circuit.

In the radioactive tracer experiments that follow, the retinas were incubated in the same solutions as in Fig. 2; that is, first in normal Ringer's solution, then in low- Ca^{2+} solution, and then in low- Ca^{2+} plus ouabain. The retinas were exposed to this last solution for 7 min before tracer addition, more than enough time for the complete suppression of the voltage responses.

Uptake of $^{22}\text{Na}^+$

Fig. 3A shows the accumulation of $^{22}\text{Na}^+$ into dark-adapted and light-exposed retinas. The retinas in both cases were exposed to the tracer for 40 s. Dark-adapted retinas accumulated $7.05 (\pm 0.75) \times 10^{-5} \text{ mmol of Na}^+/\text{mg protein}$ ($\pm \text{SEM}$; $N = 4$)², whereas light-exposed retinas accumulated only $2.72 (\pm 0.38)$

² Unless otherwise indicated, all errors are standard errors of the mean calculated with $N - 1$ weighing. Sample size, N , is the number of retinas examined.

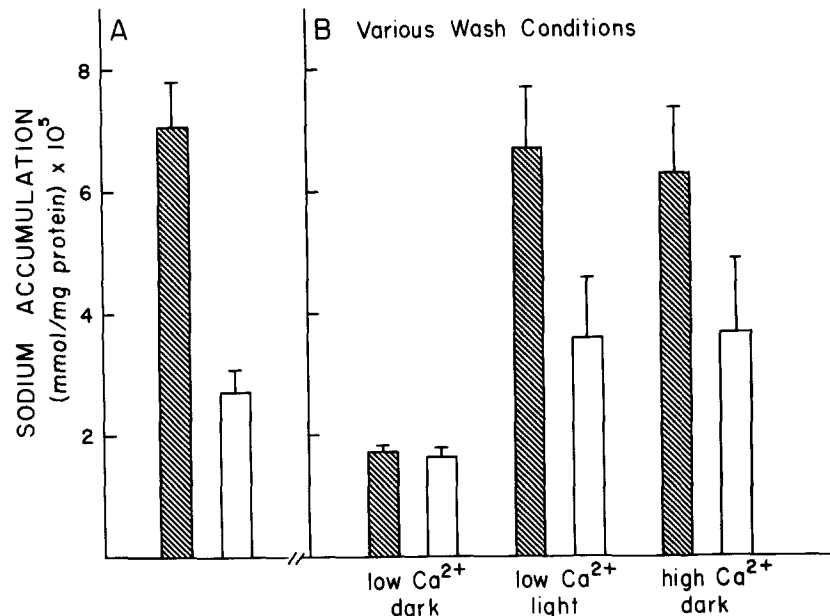


FIGURE 3. (A) Accumulation of $^{22}\text{Na}^+$ into dark-adapted (filled bars) and light-adapted (open bars) retinas. Before exposing the retinas to $^{22}\text{Na}^+$ they were incubated in solutions as follows: normal toad Ringer's solution (solution A), 10 min; low- Cl^- Ringer's solution (solution B), 2 min; low- Cl^- , low- Ca^{2+} Ringer's solution (solution C), 3 min; and then low- Cl^- , low- Ca^{2+} Ringer's solution with 0.5 mM ouabain (solution D) for 7 min. Retinas were exposed to $^{22}\text{Na}^+$ (15–25 $\mu\text{Ci/ml}$) for 40 s. In the light-exposed retinas the radioactivity was added 10 s after turning on a continuous bright light that bleached 3.9×10^6 rhodopsin molecules/receptor \cdot s. Incubation was terminated by removing the radioactivity and then washing the retinas with a 20 mM Ca^{2+} Ringer's solution (solution H) as described in the Methods. All retinas, both dark and light incubated, were exposed to the unattenuated light during the washing procedure. The data represent the mean (\pm SEM) of four dark-incubated retinas and three light-incubated retinas. (Dark retinas: 6.82, 6.73, 5.78, and 8.88×10^{-5} mmol Na^+ /mg protein; and light retinas: 2.22, 2.73, and 3.25×10^{-5} mmol Na^+ /mg protein.) The average protein content for each retina was $390 \pm 24 \mu\text{g}$ (mean \pm SEM, $N = 7$). Both the standard deviation and standard error were calculated with $N - 1$ weighing. The difference between the dark- and light-incubated retinas is significant at the 0.01 level, using the one-tailed Student's t test. (B) The effect of varying Ca^{2+} and light during the washing procedure on $^{22}\text{Na}^+$ accumulation. Retinas were prepared and exposed to $^{22}\text{Na}^+$ as in A. The first set of histograms represents the amount of $^{22}\text{Na}^+$ found in retinas washed in low- Ca^{2+} -ouabain Ringer's solution (solution D) in total darkness ($N = 3$ for dark retinas, $N = 2$ for light retinas). The middle set and last set of histograms show the sodium found in retinas washed in solution D in the presence of a bright light ($N = 3$ dark retinas; $N = 4$ light retinas) and in 20 mM Ca^{2+} solution (solution H) in darkness ($N = 3$ dark retinas; $N = 2$ light retinas), respectively. All histograms are the mean \pm SEM. The differences between the accumulations of $^{22}\text{Na}^+$ in the dark-incubated retinas washed in either light or high Ca^{2+} and the dark-incubated retinas washed in low Ca^{2+} in darkness was significant at the 0.01 level (one-tailed Student's t test).

$\times 10^{-5}$ mmol Na^+ /mg protein ($N = 3$). The magnitude of this light-dark difference was not significantly changed when 5 mM Na aspartate was included in the low- Ca^{2+} -ouabain Ringer's solution: dark retinas accumulated $7.00 (\pm 0.58) \times 10^{-5}$ mmol Na^+ /mg protein ($N = 2$) and light-exposed retinas accumulated, $3.02 (\pm 0.68) \times 10^{-5}$ mmol Na^+ /mg protein ($N = 2$). Aspartate causes a loss of responses to light in cells postsynaptic to the photoreceptor (Sillman et al., 1969; Brown and Pinto, 1974). The lack of a change in light-dependent accumulation with aspartate suggests that, as expected, the postsynaptic cells do not make a significant contribution to the light-dependent $^{22}\text{Na}^+$ uptake in low- Ca^{2+} -ouabain Ringer's solution.

The light-dependent accumulation of $^{22}\text{Na}^+$ is dependent on the procedure used to wash the retinas after exposure to radioactivity. In the experiments of Fig. 3A, the retinas were washed in high Ca^{2+} (20 mM) in the presence of bright illumination. We used a high- Ca^{2+} concentration in the wash because Ca^{2+} has been shown to block the light-dependent conductance in photoreceptors (Yoshikami and Hagsins, 1973), and we supposed that high Ca^{2+} would prevent tracer that accumulated in the photoreceptors during the incubation with radioactive solution from leaking out during washing. To ensure that the light-dependent conductance was blocked during washing, we also exposed the retinas to unattenuated illumination. Fig. 3B shows that our supposition may have been correct. If retinas are washed with low Ca^{2+} in darkness, the light-dependent uptake of $^{22}\text{Na}^+$ cannot be observed. Washing with either high Ca^{2+} in darkness or low Ca^{2+} in light preserves accumulated radioactivity. Ca^{2+} and light appear to inhibit $^{22}\text{Na}^+$ efflux.

Effect of Gramicidin D on $^{22}\text{Na}^+$ Accumulation

As a control for these experiments, we incubated retinas in the Na^+/H^+ ionophore gramicidin D before exposing them to $^{22}\text{Na}^+$. Fig. 4 shows the uptake of $^{22}\text{Na}^+$ in dark-adapted and light-exposed retinas after 80 s of incubation, with and without prior exposure to gramicidin. Accumulations without the ionophore were $8.10 (\pm 0.40) \times 10^{-5}$ mmol Na^+ /mg protein and $5.05 (\pm 0.45) \times 10^{-5}$ mmol Na^+ /mg protein for dark and light retinas, respectively. With gramicidin D, both the dark and light retinas accumulated $^{22}\text{Na}^+$ to nearly the same extent ($\sim 5 \times 10^{-5}$ mmol Na^+ /mg protein). Notice that the accumulation in gramicidin-treated retinas is closer to the value obtained in the illuminated retinas without gramicidin rather than the dark-adapted retinas. The reason for this may be that the ionophore allows transmembrane equilibration of $^{22}\text{Na}^+$ during both the initial exposure to radioactivity and during washing. Thus the ionophore may facilitate the removal of $^{22}\text{Na}^+$ from the photoreceptors during washing, when the light-dependent channels are presumably blocked by high Ca^{2+} and light.

Time Course of $^{22}\text{Na}^+$ Accumulation

The time dependence for $^{22}\text{Na}^+$ accumulation into dark-adapted (●) and light-exposed (○) retinas is shown in Fig. 5. There appear to be at least two components to the accumulation: a slow component present in both dark- and light-incubated retinas, and a fast component, present only in the dark.

The slow component is clearly time dependent, rising initially at a constant rate but saturating at $\sim 3 \times 10^{-4}$ mM Na⁺/mg protein within 12 min. The fast component, present only in the dark, appears to have reached its maximum value by 10 s, the fastest we could measure tracer uptake with our

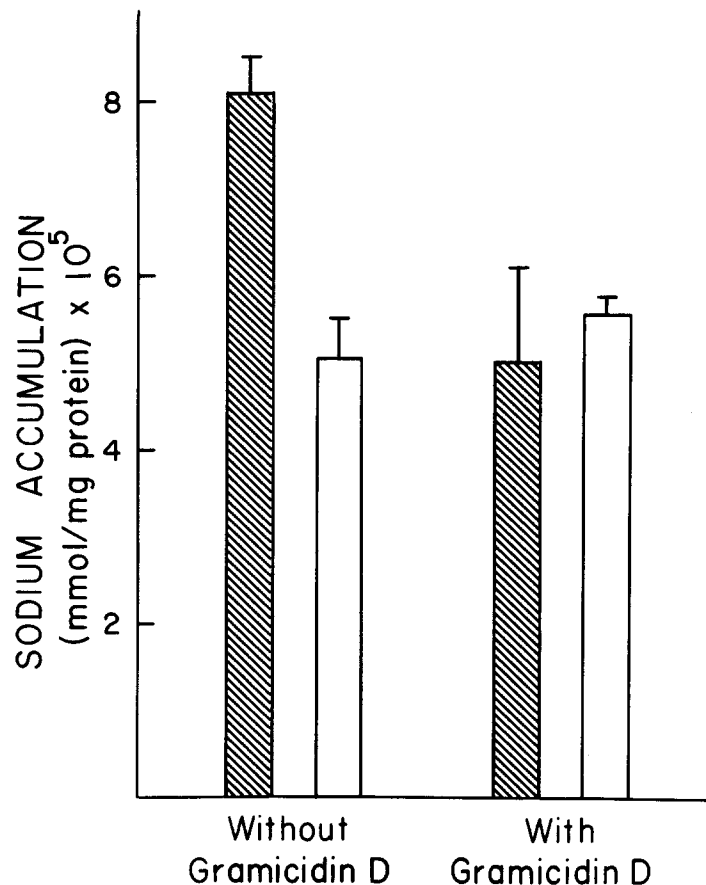


FIGURE 4. The effect of gramicidin D on ²²Na⁺ accumulation. Retinas were treated as in Fig. 3A except that they were exposed to ²²Na⁺ (15–25 μCi/ml) for 80 instead of 40 s. The retinas exposed to gramicidin D were incubated in 20 μM gramicidin D (in 0.5% ethanol, final concentration) for 10 min before adding the radioactivity. Adding ethanol in the absence of gramicidin had no effect on sodium accumulation. The results are the mean (±SEM) of three retinas in each of the four conditions and the difference between the accumulations in dark- and light-incubated retinas without gramicidin is significant at the 0.01 level (one-tailed Student's *t* test).

technique. Assuming (a) that the extra uptake of ²²Na⁺ in these dark retinas is uptake into photoreceptors, (b) that 80% of this is due to rods rather than cone receptors (by volume; Fain, 1976), and (c) that equilibration is complete in 10 s, we calculate that the rod Na⁺ influx must be at least 10⁹ Na⁺ per receptor per second. This is approximately the value of the receptor dark

current obtained by direct measurement (Zuckerman, 1973; Yau et al., 1977; and Greenblatt, 1982) or by an osmotic swelling technique (Korenbrod and Cone, 1972).

Fig. 5 also shows that after 5 min we were no longer able to measure a significant difference between the accumulation of $^{22}\text{Na}^+$ into dark- and light-incubated retinas. The reason for this may be that $^{22}\text{Na}^+$ equilibrates across the cell membranes of light-exposed photoreceptors after long exposures.

Effects of Ca^{2+} and IBMX

Fig. 6 shows that increasing the Ca^{2+} concentration from 10^{-8} to 1.8×10^{-3} M decreased $^{22}\text{Na}^+$ accumulation in dark-adapted retinas to such an extent that it abolished the light-dark difference. This experiment, together with the

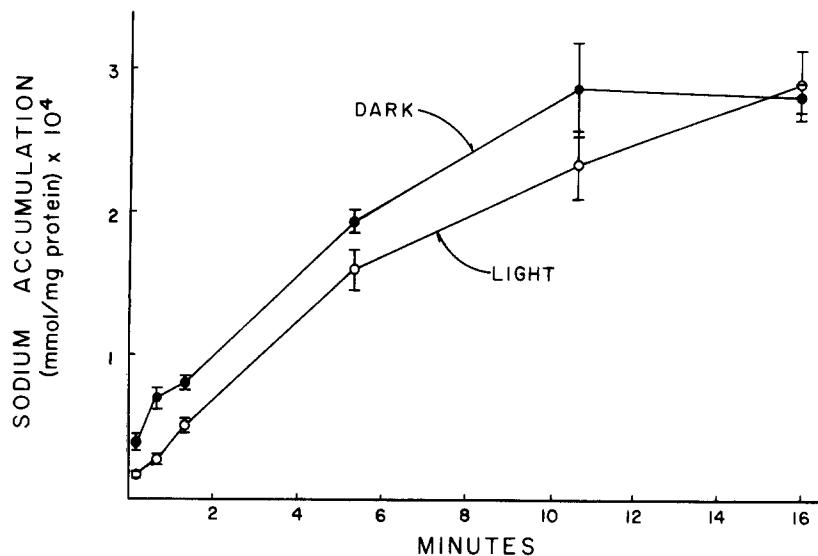


FIGURE 5. Time course of $^{22}\text{Na}^+$ accumulation in dark-adapted (●) and light-adapted (○) retinas. Retinas were prepared and exposed to radioactivity as in Fig. 3A. For the incubations with radioactivity longer than 80 s, a slow stream of oxygen was blown over the incubation solution. Light-adapted retinas were exposed to continuous light bleaching 3.9×10^6 rhodopsin molecules/receptor-s, $\sim 0.10\%$ of the pigment each second. The 16-min exposure bleached $\sim 90\%$ of the total pigment. The data represent the mean (\pm SEM) for two or more determinations at each time point.

washing experiments of Fig. 3B, suggests that Ca^{2+} can block the light-dependent influx and efflux of Na^+ into the photoreceptors. Note that in these experiments, *normal* levels of extracellular Ca^{2+} are able to suppress the light-dependent permeability. In fact, increasing extracellular Ca^{2+} levels from 10^{-8} to only 10^{-7} M eliminated the light-dark difference in $^{22}\text{Na}^+$ uptake (data not shown). The reason for this may be that the photoreceptors were exposed to ouabain, which may have had the effect of decreasing the ability of the photoreceptors to regulate intracellular levels of Ca^{2+} (see Discussion).

Fig. 6 also shows that retinas in 1.8×10^{-3} M Ca^{2+} can show a light-dependent uptake of $^{22}\text{Na}^{+}$ if the phosphodiesterase inhibitor IBMX is included in the incubation solution. This result is consistent with the experiments of Brodie and Bownds (1976), who used osmotic swelling of isolated rod outer segments to show that phosphodiesterase inhibitors increase the light-dependent permeability of the plasma membrane.

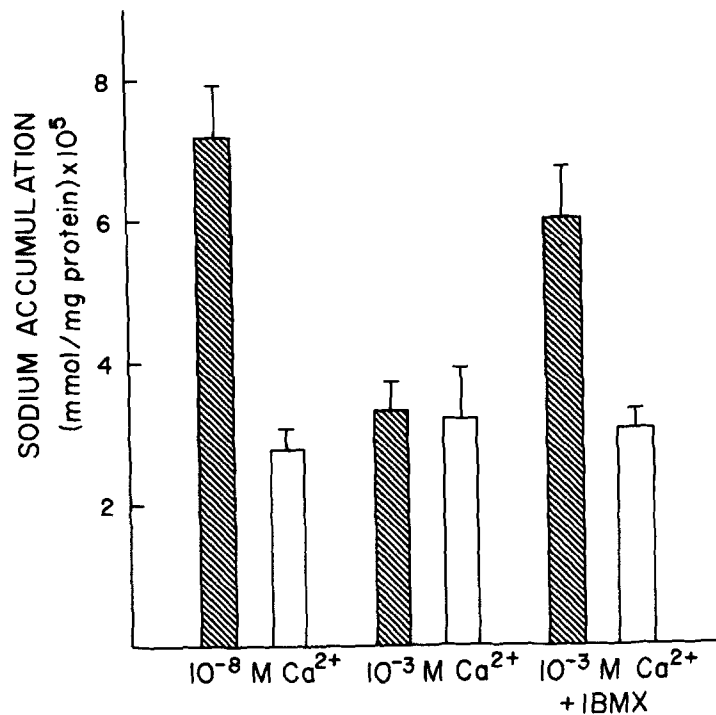


FIGURE 6. Effect of Ca^{2+} and IBMX on $^{22}\text{Na}^{+}$ accumulation. Data for the accumulation of $^{22}\text{Na}^{+}$ in 10^{-8} M Ca^{2+} are from Fig. 3A. The middle set of histograms shows the accumulation of sodium into dark-adapted (filled bar) and light-adapted (open bar) retinas incubated in normal Ca^{2+} (1.8 mM; labeled 10^{-3} Ca^{2+}). Before adding radioactivity, these normal- Ca^{2+} retinas were incubated with normal toad Ringer's solution (solution A) for 10 min, low- Cl^{-} Ringer's solution (solution B) for 2 min, and then solution B with 0.5 mM ouabain added for 7 min. Ouabain eliminated photoreceptor light responses in normal Ca^{2+} (data not shown) as it does in low Ca^{2+} (Fig. 2). The last set of histograms shows the $^{22}\text{Na}^{+}$ uptake into retinas treated similarly to the retinas in normal Ca^{2+} (middle set of histograms) except that they were exposed to 5 mM IBMX for the last 3–4 min of the 7-min incubation with ouabain. The data represent the mean (\pm SEM) of three retinas (dark- and light-incubated retinas in normal Ca^{2+}), four retinas (light-incubated retinas in normal Ca^{2+} -IBMX), or six retinas (dark-incubated retinas in normal Ca^{2+} -IBMX). The differences in $^{22}\text{Na}^{+}$ accumulation between dark- and light-incubated retinas exposed to low Ca^{2+} and normal Ca^{2+} -IBMX are significant at the 0.01 level (one-tailed Student's *t* test).

Selectivity of the Light-dependent Channel

Fig. 7 shows the uptake of $^{22}\text{Na}^+$, $^{42}\text{K}^+$, $^{86}\text{Rb}^+$, $^{131}\text{Cs}^+$, [^{14}C]methylamine, [^3H]choline, and [^{14}C]tetraethylammonium into dark-adapted and light-adapted retinas. The ordinate, which represents the amount of cation uptake in moles per milligram protein after 80 s of incubation with the tracer, is divided by the concentration of the cation in the incubation solution. Although each

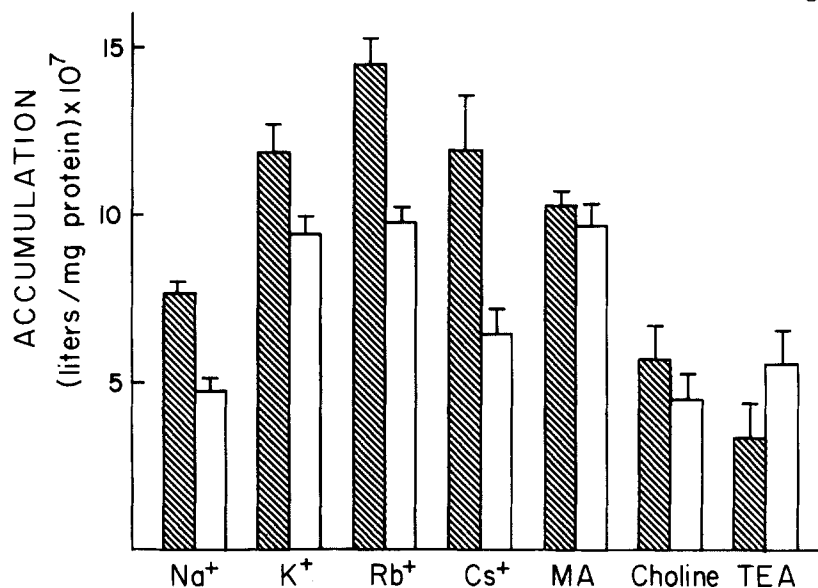


FIGURE 7. The light-dependent accumulation of alkali metal and organic cations. After retinas were exposed to low- Ca^{2+} -ouabain Ringer's solution, as described in Fig. 3A, they were incubated with either $^{22}\text{Na}^+$ (141 $\mu\text{Ci}/\text{mM}$), $^{42}\text{K}^+$ (400 $\mu\text{Ci}/\text{mM}$), $^{86}\text{Rb}^+$ (91 mCi/mM), $^{131}\text{Cs}^+$ (84 mCi/mM), [^{14}C]methylamine (61 mCi/mM), [^3H]choline (80 Ci/mM), or [^{14}C]tetraethylammonium (4.4 mCi/mM) at a total concentration as follows: 110 mM Na^+ ; 2.5 mM K^+ ; 0.45 mM Rb^+ ; 0.15 mM Cs^+ ; 0.12 mM methylamine; 0.25 mM choline; and 0.23 mM tetraethylammonium. Retinas were incubated for 80 s either in the dark (filled bars) or in the presence of a light bleaching 3.9×10^6 rhodopsin molecules/receptor \cdot s (open bars). Tracer accumulation was converted to liters per milligram protein by dividing radioactivity accumulated by the specific activity and the concentration. Each histogram represents the mean (\pm SEM) for three or more retinas with the numbers of retinas examined in each case, for dark- and light-incubated retinas, respectively: Na^+ (3, 3); K^+ (10, 10); Rb^+ (4, 3); Cs^+ (4, 3); methylamine (5, 3); choline (3, 3); and tetraethylammonium (5, 5). The differences between the accumulation of cations in dark- and light-exposed retinas for Na^+ , K^+ , Rb^+ , and Cs^+ are significant at the 0.01 level (one-tailed Student's t test). The data for Na^+ are replotted from Fig. 4.

cation shows a varying amount of nonspecific accumulation, the alkali metal cations show a significant light-sensitive uptake (one-tailed Student's t test, $P = 0.01$), whereas the organic cations do not. A significant light-sensitive accumulation of $^{204}\text{Tl}^+$ also can be obtained in toad retina, but the results are not presented in Fig. 7 because the nonspecific uptake of Tl^+ was much greater

than is shown for the other cations. Expressed in the permeability units used in the ordinate of Fig. 7, dark-adapted retinas accumulated $3.02 (\pm 0.19) \times 10^{-6}$ liter/mg protein of TI^+ , whereas light-adapted retinas accumulated $2.13 (\pm 0.24) \times 10^{-6}$ liter/mg protein during 80 s of incubation.

Fig. 8 shows that Ca^{2+} and IBMX have an effect on the light-dependent $^{131}\text{Cs}^+$ accumulation that is similar to the effect observed with $^{22}\text{Na}^+$ (Fig. 6).

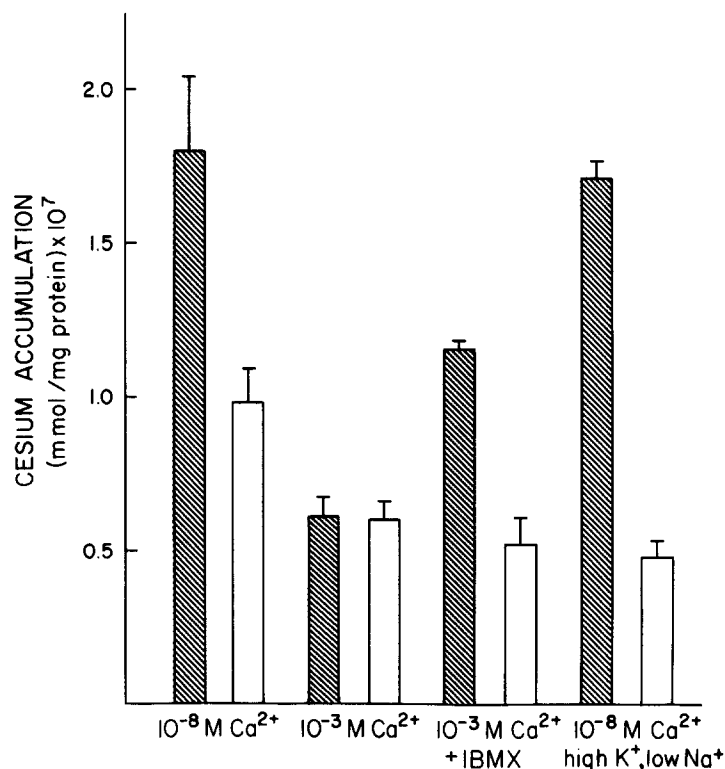


FIGURE 8. The effect of Ca^{2+} , IBMX, and high- K^+ -low- Na^+ solution on $^{131}\text{Cs}^+$ accumulation. Data for uptake of Cs^+ in low Ca^{2+} (10^{-8} M) are replotted from Fig. 7. The experiments in normal Ca^{2+} (1.8 mM; labeled 10^{-3} M Ca^{2+}) and normal Ca^{2+} plus IBMX were performed similarly to the experiments shown in Fig. 6, except that $^{131}\text{Cs}^+$ was used instead of $^{22}\text{Na}^+$. The last set of histograms shows that a significant light-dependent uptake can be observed in high- K^+ -low- Na^+ Ringer's solution. For this experiment the retinas were incubated in the following solutions before adding the radioactivity: normal toad Ringer's solution (solution A) for 10 min; high- K^+ -low- Na^+ solution (solution E), 2 min; high- K^+ -low- Na^+ , low- Ca^{2+} Ringer's solution (solution F), 3 min; and then solution F with 0.5 mM ouabain (solution G), 7 min. The final Cs^+ concentration was 0.15 mM in the last two solutions in all the experiments and the specific activity was between 80 and 115 mCi/mM. The data represent the mean (\pm SEM) of three retinas (dark- and light-exposed retinas in normal Ca^{2+} and high K^+ -low Na^+) or four retinas (dark- and light-exposed retinas in normal Ca^{2+} -IBMX), and the differences between the dark- and light-exposed retinas in low Ca^{2+} , normal Ca^{2+} -IBMX, and high K^+ -low Na^+ are significant at the 0.01 level (one-tailed Student's *t* test).

Ca^{2+} blocked the light-dependent $^{131}\text{Cs}^+$ accumulation, and IBMX restored this uptake. This suggests that Cs^+ , and perhaps the other alkali metal ions as well, permeate the photoreceptors through the same conductance pathway as Na^+ .

Cahalan and Begenisich (1976) demonstrated that the selectivity of the voltage-sensitive Na^+ channel in squid axon is altered by a decrease in cytosol K^+ concentration. If the photoreceptor light-dependent conductance were similarly affected, then ouabain treatment, which would have blocked the Na^+, K^+ pump and thus reduced $[\text{K}^+]_i$, may have changed the conductance

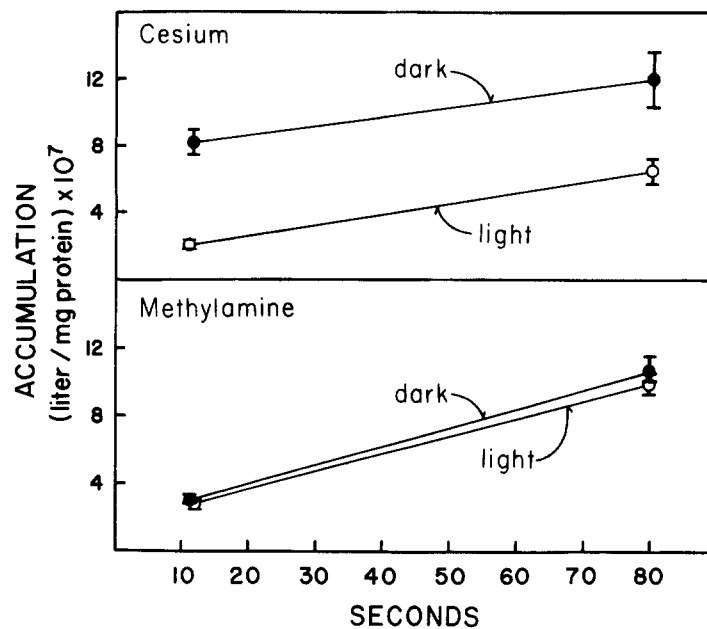


FIGURE 9. Time course of $^{131}\text{Cs}^+$ and $[^{14}\text{C}]$ methylamine accumulations. The data for 80 s are from Fig. 7. The specific activities and final concentrations of Cs^+ and methylamine were the same for the 10–12-s time point as for 80 s (see Fig. 7). The data represent the mean (\pm SEM) of two or more retinas.

selectivity in our experiments. As a control for this possibility, we incubated the retinas in Ringer's solutions whose cation compositions are approximately equivalent to the internal milieu (solutions E–G; see Owen and Torre, 1981). These solutions contained 28 mM Na^+ and 90.5 mM K^+ but were otherwise identical to those used in the experiments above. Since $[\text{K}^+]$ would have been nearly the same inside and outside the rod in these solutions, ouabain in treatment could not have produced large changes in the intracellular levels of K^+ . However, the last set of histograms in Fig. 8 shows that a significant light-dark difference can still be observed for $^{131}\text{Cs}^+$ under these conditions.

The time courses for $^{131}\text{Cs}^+$ and $[^{14}\text{C}]$ methylamine accumulations into dark-adapted and light-adapted retinas are shown in Fig. 9. Like $^{22}\text{Na}^+$, $^{131}\text{Cs}^+$

shows a slow component present in both dark- and light-adapted retinas, and a fast component present only in dark-adapted retinas. Because $^{131}\text{Cs}^+$ has a light-dark difference at 10 s that is similar to the difference at 80 s, equilibration of $^{131}\text{Cs}^+$ into photoreceptors must have taken place in <10 s. [^{14}C]-methylamine shows only the slow uptake component. In an experiment not illustrated in this figure, we extended the incubation time with [^{14}C]methylamine to 5 min, 30 s. After this time a slightly larger light-dependent uptake was obtained with dark-adapted retinas accumulating $23.5 (\pm 1.2 \times 10^{-7})$ liter/mg protein and light-adapted retinas accumulating $20.9 (\pm 1.1 \times 10^{-7})$ liter/mg protein; however, this difference still was not significant (one-tailed Student's t test, $P = 0.01$).

DISCUSSION

Evidence That We Are Measuring the Light-dependent Conductance

We believe that the tracer assay described in this report measures the uptake of ions through the photoreceptor light-dependent channel. Our evidence for this is as follows. First, light-dependent uptake and efflux of tracer are assayed in retinas exposed to low- Ca^{2+} -ouabain Ringer's solution. In this medium, the photoreceptor light-sensitive conductance continues to open and close (Fig. 2), but other channels in photoreceptors and secondary nerve cells or glia are unlikely to have been affected by light. Second, light, which leads to a closing of the light-sensitive channels, blocks accumulation of $^{22}\text{Na}^+$ (Fig. 3A) and inhibits $^{22}\text{Na}^+$ efflux from retinas exposed to the tracer in darkness (Fig. 3B). Third, gramicidin D, which should facilitate $^{22}\text{Na}^+$ movements across cell membranes, abolishes the light-dependent uptake of $^{22}\text{Na}^+$ (Fig. 4). Fourth, the rate of light-dependent uptake and equilibration is rapid enough to be explained by the photoreceptor light-sensitive channels (Fig. 5). Fifth, Ca^{2+} , which blocks the light-dependent channels in photoreceptors, also blocks the uptake of $^{22}\text{Na}^+$ (Fig. 6) and the efflux of $^{22}\text{Na}^+$ (Fig. 3B). Finally, IBMX, which increases the light-dependent permeability, increases the uptake of $^{22}\text{Na}^+$ (Fig. 6).

Several observations eliminate the possibility that the light-dependent accumulations of $^{22}\text{Na}^+$ that we obtain could have been caused by light-induced changes in nonspecific binding or photoreceptor volume. If a change in binding or volume were responsible for the light-dependent accumulation, light would either inhibit uptake and facilitate the apparent efflux of radioactivity or promote uptake and inhibit washout. We find, however, that light blocks tracer uptake and tracer efflux, a result much more easily explained by a gating mechanism. In addition, all retinas were exposed to illumination for >3 min during the washing procedure, and this probably would have equalized any nonspecific light-induced effects in our samples. Finally, both light-induced changes in surface potential at photoreceptor membranes (Cafiso and Hubbell, 1980) and changes in photoreceptor volume (see Falk and Fatt, 1972) are small and in a direction opposite to those required to explain our observed light effect. The most reasonable mechanism for our light-dependent accumulation is that radioactivity is actually accumulating inside retinal cells across cell membranes, and that in our condition of low- Ca^{2+} -ouabain, the

photoreceptors are the primary source for light-dependent uptake. Both the abolition of the light-dependent accumulation by gramicidin D and the lack of effect of aspartate on the magnitude of the light-dependent accumulation are consistent with this interpretation.

Caretta et al. (1979) have demonstrated that the efflux of $^{42}\text{K}^+$, $^{86}\text{Rb}^+$, and $^{22}\text{Na}^+$ (but not $^{36}\text{Cl}^-$) from loaded photoreceptor membranous disks is increased in a dose-dependent manner by cyclic GMP. Since in our experiments we have measured changes in the uptake of radioactivity in low Ca^{2+} or normal Ca^{2+} with IBMX, both of which increase intracellular cyclic GMP (see Woodruff and Fain, 1982), it is possible that our measurements reflect to some extent the permeability of the rod disks. The disks, however, cannot be entirely responsible for the light-dependent accumulation that we observe. There is not enough osmotically active volume inside disks to account for the magnitude of the light-dark difference we obtain. The disk volume for a single rod rod outer segment is $\sim 1.4 \times 10^{-13}$ liters, whereas we calculate, assuming internal sodium to be between 55 and 110 mM, that the "volume" occupied by the light-dependent radioactivity is 3.4×10^{-13} – 6.7×10^{-13} liters (data from Fig. 3A). Similar calculations with Cs^+ , Rb^+ , and Tl^+ yield similar results. All give volumes greater than the volume of the disks, but smaller than the osmotically active volume of an entire rod, which is $\sim 2 \times 10^{-12}$ liters. That the magnitude of the light-dependent cation uptake would convert to a volume smaller than the rod is reasonable, since some of the light-dark difference would probably have been dissipated during the 3-min washing procedure.

In a separate set of experiments, using isolated rod outer segments from frog retinas, Cavaggioni et al. (1973) demonstrated an efflux of $^{42}\text{K}^+$ and $^{86}\text{Rb}^+$ from isolated rod outer segments that was decreased by illumination. They also observed that the $^{42}\text{K}^+$ and $^{86}\text{Rb}^+$ effluxes were inhibited by increasing extracellular $[\text{Ca}^{2+}]$ or decreasing extracellular $[\text{Na}^+]$ —two treatments that probably increase intracellular $[\text{Ca}^{2+}]$ and thus block the light-dependent conductance. Although they suggested that the light inhibition of efflux might be the result of increased retention of the positively charged species caused by light-induced hyperpolarization of the isolated outer segments, it seems more reasonable now to suggest that they were studying the permeability of the photoreceptor light-dependent channel.

Although we are unable, with our method, to measure the initial rate of tracer uptake and therefore cannot give the magnitude of the photoreceptor light-dependent permeability, our rough estimate that at least 10^9 Na ions must be entering each rod photoreceptor per second is similar to the estimates made by Korenbrot and Cone (1972) using the rate of osmotic swelling in hyperosmotically shocked rod outer segments from frog ($\sim 2.1 \times 10^9 \text{ Na}^+/\text{rod} \cdot \text{s}$). Zuckerman (1973), Yau et al., (1977), and Greenblatt (1982) obtained a current of 1 – 2×10^8 Na ions entering each rod each second, a value ~ 1 log unit less than our estimate. However, these measurements were made at millimolar Ca^{2+} levels. In the low- Ca^{2+} solutions that we used, the magnitude of the dark current can increase 10-fold or more (Yau et al., 1981; Greenblatt, 1982).

Effect of Ca²⁺ and IBMX

The second set of histograms in Fig. 6 shows that we were unable to obtain a light-dependent uptake of ²²Na⁺ into retinas exposed to normal [Ca²⁺]_o (1.8 × 10⁻³ M). A similar result is obtained with [Ca²⁺]_o as low as 10⁻⁷ M. This may seem surprising since the Na⁺ flux might be thought to be large enough at these Ca²⁺ concentrations to produce rapid equilibration in dark-adapted photoreceptors. However, our incubation solution contained 0.5 mM ouabain, which should have had the effect of collapsing the sodium gradient. Since rods appear to rely upon Na⁺/Ca²⁺ countertransport to remove cytosol Ca²⁺ (Schnetkamp, 1980; and see Fain and Lisman, 1981), photoreceptors exposed to ouabain would presumably be less able to remove intracellular Ca²⁺ and therefore more sensitive to changes in extracellular [Ca²⁺]. The restoration of a light-dependent uptake of ²²Na⁺ when the phosphodiesterase inhibitor IBMX is added to the incubation solution in the presence of normal Ca²⁺ levels suggests that increasing cyclic nucleotide levels in photoreceptors can lead to an increase in light-dependent permeability. In the following report (Woodruff and Fain, 1982), we show that under conditions parallel to those shown in Fig. 6, retinal cyclic GMP levels are increased from ~40 pmol cGMP/mg protein to ~150 pmol cGMP/mg protein by IBMX. An increase in cyclic GMP might directly increase the permeability of the light-dependent conductance by binding to the conductance mechanism or by regulating enzyme activities that modify the permeability (see Bownds, 1981). Alternatively, increases in cyclic GMP might increase the light-dependent permeability indirectly by decreasing intracellular Ca²⁺ levels (Yoshikami and Haggins, 1971). Recently, George and Haggins (1981) have suggested that cyclic GMP can influence the Ca²⁺ transport properties of photoreceptor disk membranes, such that increasing cyclic GMP increases Ca²⁺ uptake by the disks.

Ion Selectivity of the Light-dependent Conductance

Our experiments show that the light-dependent channel is permeable to K⁺, Rb⁺, Cs⁺, and Tl⁺ in addition to Na⁺. Since we obtained significant light-dark differences for both Cs⁺ and Na⁺ either in 10⁻⁸ M Ca²⁺ alone or in normal Ca²⁺ in the presence of IBMX, it seems unlikely that our results are significantly affected by any changes in the channel selectivity in low-Ca²⁺ solutions. Also, we find that the light-suppressible accumulation of Cs⁺ is unchanged when measured in a Ringer's solution whose composition approximates that of the photoreceptor cytosol (Fig. 8), which indicates that changes in the selectivity of the channel produced by altered intracellular Na⁺ and K⁺ concentrations that might occur after ouabain treatment could not have been responsible for the light-dark difference for Cs⁺.

We cannot, unfortunately, suggest a relative selectivity for the metal cations from the data in our experiments. An analysis of that sort depends upon an accurate determination of the initial rate of radioactive uptake, and the time courses of ²²Na⁺ (Fig. 5) and ¹³¹Cs⁺ (Fig. 9) accumulation indicate that our measurements are equilibrium values. Our results are consistent with the evidence presented by Yau et al. (1981) that the cations Li⁺, K⁺, Rb⁺, and

Cs⁺ can to some extent permeate the light-dependent channel. Furthermore, neither we nor Yau et al. (1981) could observe a significant permeation of choline, and we also were unable to see a significant light-dependent influx of other organic cations (methylamine or TEA, Fig. 7). In our experiments, we cannot eliminate the possibility that light stimulated the uptake of choline or the other organic cations into the photoreceptors through a mechanism independent of the light-dependent conductance, or that the passage of the organics in their neutral form could have been large enough to equilibrate the radioactivity of these species in both light and darkness. However, the apparent agreement of our results with those of Yau et al. (1981) strongly suggests that organic cations cannot permeate the light-dependent conductance.

Our results show that the light-dependent conductance of rods must be at least 3.4 Å in diameter, the unhydrated diameter of Cs⁺ (Hille, 1975). Our studies, together with those of Yau et al. (1981), suggest that the light-dependent conductance is unlikely to resemble the acetylcholine-sensitive channel at the motor endplate or the Na⁺ channel of epithelia. The exact nature of its ion selectivity will have to await further experimentation.

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