The Effects of Intracellular Iontophoretic Injection of Calcium and Sodium Ions on the Light Response of *Limulus* Ventral Photoreceptors

J. E. LISMAN and J. E. BROWN

From the Department of Biology and Research Laboratory of Electronics, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, and the Marine Biological Laboratory, Woods Hole, Massachusetts 02543. Dr. Brown's present address is the Department of Anatomy, Vanderbilt University School of Medicine, Nashville, Tennessee 37203.

ABSTRACT During intracellular iontophoretic injection of Ca^{++} into *Limulus* ventral photoreceptor cells, there is a progressive diminution of the light response. Following Ca^{++} injection, the size of the light response slowly recovers. Similarly, there is a progressive diminution of the light response during intracellular injection of Na^{+} and recovery after the injection is stopped. The rate of diminution during Na^{+} injection is greater for higher $[Ca^{++}]_{out}$. In solutions which contain 0.1 mM Ca^{++}, there is nearly no progressive decrease in the size of the light response during Na^{+} injection. Intracellular injections of Li^{+} or K^{+} do not progressively decrease the size of the light response. We propose that an increase in $[Na^{+}]_{in}$ leads to an increase in $[Ca^{++}]_{in}$ and that an increase in $[Ca^{++}]_{in}$ by any means leads to a reduction in responsiveness to light.

INTRODUCTION

Limulus photoreceptor cells respond to illumination with a positive-going (depolarizing) change in membrane voltage. This depolarizing receptor potential often has a large transient phase followed by a smaller plateau which persists until illumination ends (Tomita, 1956; Fuortes, 1958). The mechanisms generating the receptor potential have been studied by changing the external ionic environment of the photoreceptors. These studies have concluded, for both lateral and ventral eye photoreceptors of *Limulus*, that there is a light-induced increase in conductance of the cell membrane to sodium ions; sodium ions flow into the cell down their electrochemical gradient, depolarizing the membrane. (Kikuchi et al., 1962; Millecchia and Mauro, 1969 *b*; Brown and Mote, 1971). These conclusions were based on the observations that reducing the extracellular concentration of sodium

ions ($[Na^+]_{out}$) leads to both a reduction in the amplitude of the light response and a more negative value of reversal potential of the light response.

In contrast, reducing the extracellular concentration of calcium ions ($[Ca^{++}]_{out}$) leads to larger amplitudes of both the receptor potentials and light-induced currents (Millecchia and Mauro, 1969 *a*, *b*). Millecchia and Mauro (1969 *b*) concluded that extracellular calcium ions did not contribute substantially (as a charge-carrying species) to the light-induced current. Instead, calcium ions acted as a "modifier" of the light-induced sodium current.

In this paper we have examined the effects of intracellular injection of sodium or calcium ions (as well as potassium, lithium, and chloride ions). Our data show that an increase in intracellular calcium ions can reduce the size of the light response. In addition we have shown that intracellular injection of sodium ions in the presence of extracellular Ca^{++} also can reduce the size of the light response. We suggest that a rise in intracellular Na^+ can lead to a rise in intracellular Ca^{++}. Thus, intracellular injection of Na^+ can indirectly (via Ca^{++}) reduce the size of the light response.

In a companion paper (Brown and Lisman, 1972) we reexamine the involvement of the "electrogenic sodium pump" in the generation of the light response.

METHODS

The ventral eye of *Limulus* (Clark et al., 1969; Demoll, 1914) was dissected out of the animal and mounted in a Sylgard 184 (Dow Corning Corp., Midland, Mich.) chamber ($\frac{1}{8} \times 1\frac{1}{4}$ inches).

Artificial seawater (ASW) was continuously perfused through the chamber during the experiment by a gravity-feed system; the outflow was provided by an aspirator. The solution in the Sylgard chamber could be changed quickly; cells impaled with a microelectrode responded to a change in solution within 10–15 sec. The compositions of the seawaters used in this paper are given in Table I. Light from a tungsten filament was passed through an electromechanical shutter, a water cell, and neutral density filters and was focused on the preparation. Stimulating light flashes were given repetitively (except where noted) in order that the cell being studied remained in a relatively light-adapted condition.

Micropipettes were prepared by the technique of Tasaki et al. (1968), and were filled with 3 M KCl or 1 M NaCl; their resistances were 10–20 MΩ. For the intracellular iontophoretic injection of Ca^{++}, the micropipettes were filled with either 1 M $CaCl_2$ or with a Ca^{++}–ethylene glycol bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) buffer (P. Brandt and J. Reuben, personal communication). This Ca^{++}-EGTA solution contained 0.09 M $Ca(OH)_2$, 0.1 M EGTA, and 0.1 M tris(hydroxymethyl)aminomethane (Tris). The micropipettes used for Ca^{++} injections were more blunt than those filled with KCl or NaCl; their resistances were about 3 MΩ when filled with 3 M KCl and 40–50 MΩ when filled with the Ca^{++}-EGTA solution.

Membrane voltage was measured between a microelectrode inserted into the cell and a 3 M KCl-agar electrode placed in the seawater bath. The electronics and voltage-clamp circuit have been described elsewhere (Lisman and Brown, 1971).

RESULTS

Injection of Calcium Ions

A photoreceptor cell in the *Limulus* ventral eye can be impaled with two micropipettes, one filled with 3 M KCl, the other with a solution containing calcium ions. Using the KCl-filled electrode to monitor membrane voltage, the effects of intracellular iontophoretic injection of Ca^{++} from the other pipette can be measured. The calcium ions were passed iontophoretically out of an electrode filled either with 1 M $CaCl_2$ or with a Ca^{++}-EGTA solution. The resistance of electrodes filled with $CaCl_2$ very often became extremely high. We presume that the transport of Ca^{++} out of an electrode

TABLE I
COMPOSITION OF SEAWATER SOLUTIONS
Values are in millimoles per liter.

	NaCl	KCl	$CaCl_2$	$MgCl_2$	$MgSO_4$	$NaHCO_3$	Tris-HCl (pH 7.8–8.0)	Sucrose
ASW	423	10	10	22.1	26.2	2.2	15	—
Low-Ca^{++} SW	423	10	0.1	22.1	26.2	2.2	15	30.4
$SO_4^{=}$-free SW	368	10	10	48.3	—	—	15	114
High-Ca^{++}SW	368	10	50	48.3	—	—	15	—

diminished when this dramatic increase in resistance occurred; we terminated all experiments when the increased electrode resistance was seen. The Ca^{++}-EGTA electrodes remained reliable more often than those filled with $CaCl_2$. We obtained similar results with Ca^{++} injection out of electrodes filled with either $CaCl_2$ or Ca^{++}-EGTA solutions.

Fig. 1 shows the results of such an experiment with the preparation bathed in ASW. A 1.0 sec flash was given every 10 sec; the intensity of the flash was adjusted to elicit both transient and steady phases of the receptor potential, but was not bright enough to saturate the amplitude of the transient phase (see Fig. 11). When a constant current was passed through the Ca^{++}-EGTA–filled pipette, the membrane voltage was made more positive (depolarized) for the duration of the current injection. The amplitude of the receptor potential was progressively reduced during the injection.

When the constant current through the Ca^{++}-EGTA–filled electrode was turned off, the dark membrane voltage rapidly turned toward its value preceding the injection. The amplitude of the receptor potentials, which had become smaller during the injection of calcium ions, slowly recovered

after the injection (Figs. 1 and 2). With stimuli of moderate intensity, the size of the transient phase of the receptor potential diminished proportionately more than the size of the steady phase, as seen in Fig. 2.

The magnitude of the reduction of the receptor potential during Ca^{++} injection and the time-course of the subsequent recovery of the receptor potential depended on both the intensity of the repetitive stimuli and the size of the current injected through the Ca^{++}-EGTA–filled pipette. At a given light intensity, the rate of diminution of the receptor potential was greater, and the subsequent recovery slower, for larger injections than for smaller ones (Fig. 1 A). On the other hand, for a given size of calcium ion injection, less decrement was seen for bright flashes than for dim ones (Fig. 1 B).

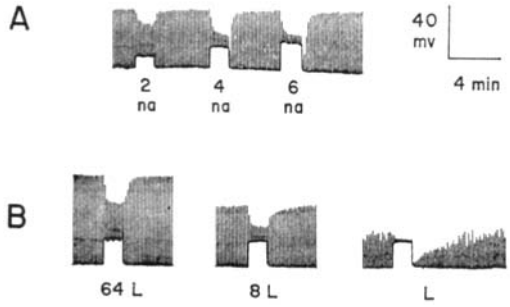

FIGURE 1. Intracellular iontophoretic injection of Ca^{++}. The membrane was depolarized by the injection of a constant current through a Ca^{++}-EGTA–filled pipette. Stimuli of 1 sec duration were given 1/10 sec. (A), stimulus intensity fixed; the magnitude of the injection current is marked below each injection interval. (B), same cell as (A). Injection current was fixed at 5 na; relative light intensity is marked below each record.

Injection of Sodium Ions

Sodium ions were injected from an intracellular NaCl-filled micropipette while simultaneously monitoring membrane voltage with a KCl-filled electrode. The injection of Na^+ produced a progressive decrement of the light response during the injection and slow recovery of the light response subsequent to the injection (Fig. 3). The decrement in the transient phase of the receptor potential was proportionately greater than that of the steady phase (Fig. 4).

In addition to the progressive decrement of the light response during the injection of Na^+, there was a pronounced hyperpolarization which often followed the injection of sodium ions (Figs. 3 D, 4, and 5). This phenomenon is treated more fully in a companion paper (Brown and Lisman, 1972). We have not observed a hyperpolarization following injection of calcium ions.

As seen previously with the injection of calcium ions, both the amount of sodium ion injected and the intensity of the light stimuli affect the size of the decrement in light response. For larger Na^+ injections at a fixed light intensity, the decrement was greater (Fig. 3); at a fixed current through the NaCl-filled electrode, the diminution was greater for lower than for higher light intensities (Fig. 5). Thus, in every regard except for the presence of an

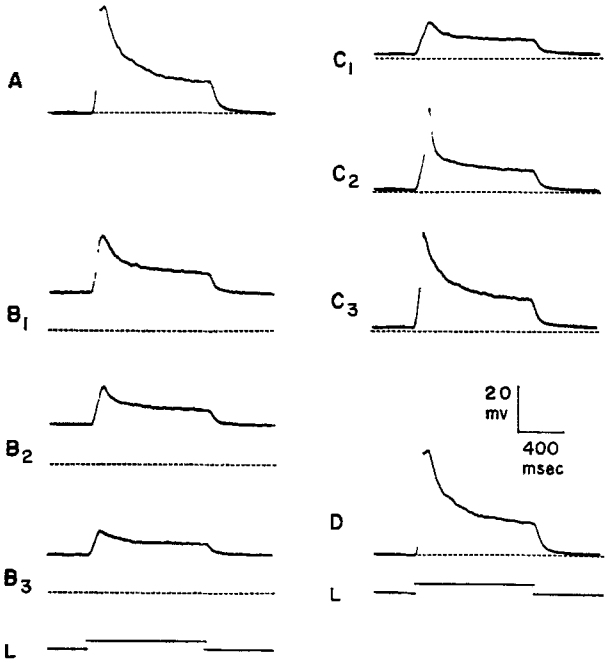

FIGURE 2. Intracellular iontophoretic injection of Ca^{++}; oscilloscope traces of the data in Fig. 1 B (8L). (A), before injection of Ca^{++}. (B_1), (B_2), (B_3), the first, fifth, and tenth responses during injection. (C_1), (C_2), (C_3), the first, second, and fourth responses following the end of injection. (D) 4 min after injection of Ca^{++}.

after-hyperpolarization, the injection of sodium ions produces the same effects as does the injection of calcium ions.

During the injection of sodium ions described above, a prolonged constant current was passed across the cell membrane; this current caused a prolonged displacement of membrane voltage. To test whether a progressive reduction of the light response could be observed in the absence of a displacement of membrane voltage, we inserted three micropipettes into a single photoreceptor. Two electrodes were filled with 3 M KCl and were used as the voltage monitoring electrode and the current-passing electrode in a voltage-clamp circuit; the third pipette was filled with 1 M NaCl and was used to inject sodium ions. All three electrodes recorded identical recep-

tor potentials, indicating that the inside of the receptor cell was equipotential. The receptor potential elicited by a brief, dim flash at the beginning of the experiment is shown in Fig. 6 A.

To record the effect of sodium injection on light-induced current (Fig. 6 C), we voltage clamped the cell, using resting voltage as holding voltage. During the injection of 5 na of current through the NaCl-filled pipette, the voltage clamp allowed no change in voltage or net dark current across the membrane (Fig. 6 B). However, the light-induced current progressively decreased during the injection and recovered following the injection (Fig. 6 B, D, and E). This entire procedure did not permanently alter the light response of the cell; for comparison, the receptor potential recorded 5 min

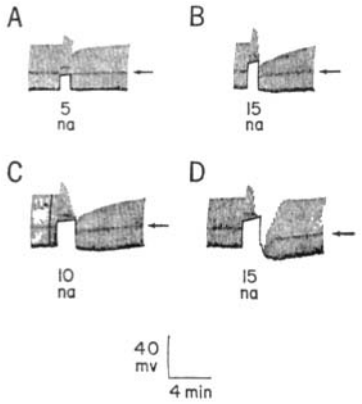

FIGURE 3. Intracellular iontophoretic injection of sodium ions. Horizontal arrows mark the amplitude of the steady phase of the receptor potential (dark band). The membrane was depolarized during the injection of a positive constant current through a 1 M NaCl–filled pipette; the magnitude of the current is marked below each trace.

after the end of the record in Fig. 6 B is seen in Fig. 6 F. These results indicate that neither a change in net current across the membrane, nor the displacement of membrane voltage is necessary for an injection of sodium ions to decrease the size of the light response.

With this three-electrode type of experiment, we also measured the reversal voltage for the light-induced current before, during, and after the injection of sodium ions, as follows. Stimuli were given once every 10 sec. The clamping voltage was slowly displaced from resting voltage to a more positive voltage. When the clamping current in the dark became steady, the light-induced current was determined. Then the clamping voltage was slowly returned to resting voltage. The procedure was repeated at successively more positive clamping voltages until the light-induced current reversed in sign (Millecchia and Mauro, 1969 *b*; Lisman and Brown, 1971).

At "reversal voltage," a small biphasic light-induced current was usually recorded (Lisman and Brown, 1971). The value of reversal voltage was about +15–20 mv.

During the sodium ion injection, the light intensity was increased to regain a light-induced signal large enough to determine its change of sign. This change in light intensity ought not to cause a change in reversal potential; Millecchia and Mauro (1969 *b*) found that reversal potential was independent of stimulus intensity. After the light intensity was increased, the

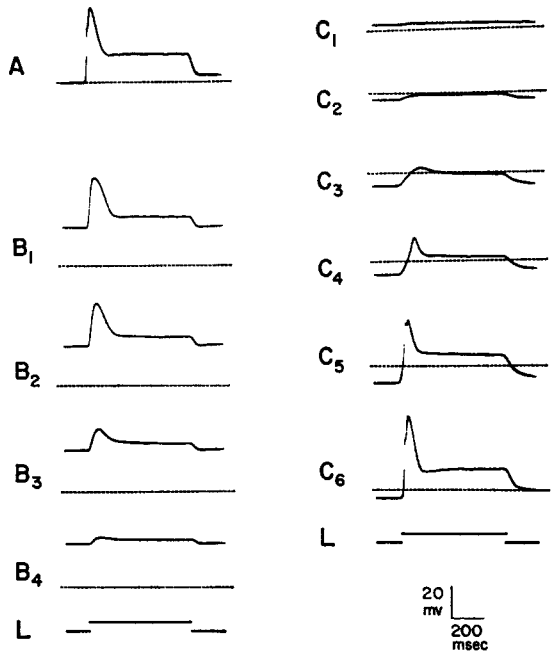

FIGURE 4. Intracellular iontophoretic injection of sodium ions recorded as in Fig. 3 D. (A), before the injection of Na^+. (B_1)–(B_4), alternate responses recorded during the injection of Na^+. (C_1) – (C_6), alternate responses recorded during recovery after the injection of Na^+. 5 min after injection. Traces marked *L* are a light monitor. The dashed line associated with each trace is drawn at the "dark" resting voltage preceding injection (as in A).

clamping voltage was displaced to the previously determined reversal voltage and the light-induced current was measured; within the error of our technique (not more than 5 mv), the reversal voltage had not changed. The measurement was repeated after the sodium injection and the value of the reversal voltage was still the same. Thus, the injection of sodium ions did not significantly change the reversal voltage of the light response, although the currents induced by fixed intensity stimuli decreased markedly.

Injection of Potassium Ions

Another indication that neither the displacement of membrane voltage nor the passage of an extrinsic current across the membrane is responsible for the decrement of the light response seen with Ca^{++} or Na^{+} injections could be obtained from the injection of K^{+}. During the injection of a constant current through a KCl-filled pipette, we did not observe any progressive decrement of the receptor potential during the injection (Fig. 7 A). The light response was smaller at the more positive membrane voltage, as expected, since the

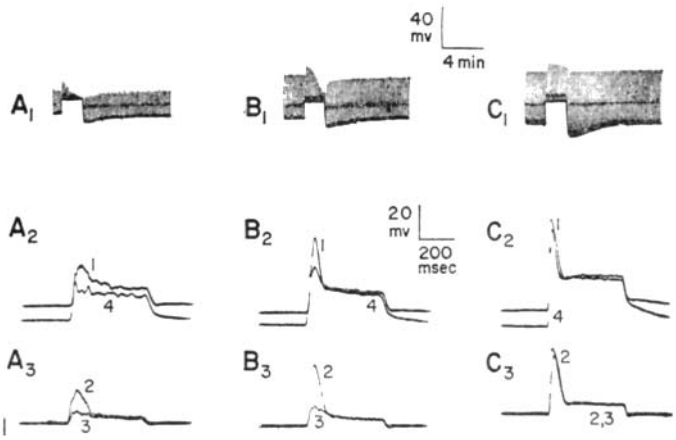

FIGURE 5. Intracellular injection of Na^{+} during repetitive stimulation at different light intensities. In (A_1), (B_1), and (C_1) +10 na was injected (for 2 min) through the NaCl-filled pipette. (A_2), (B_2), and (C_2) show receptor potentials recorded before the injection (*1*) and immediately after the injection (*4*). (A_3), (B_3), and (C_3) show receptor potentials recorded just before the end of the injection (*3*). The light intensity in (B) was 16 times that in (A); the light intensity in (C) was 256 times that in (A).

voltage was closer to the reversal voltage for the light response (Millecchia and Mauro, 1969 *b*).

The first light response subsequent to a large positive displacement of membrane voltage was smaller than the light response elicited by an identical stimulus given before the depolarization. This is shown in the voltage-clamp records in Fig. 8; the light-induced current after the voltage displacement is smaller than that before it. In this experiment (Fig. 8 C and D), the voltages to which the cell was clamped were −7 and +21 mv. These voltages were either in or near the negative resistance region of the current-voltage (*I–V*) relation of the cell membrane (Lisman and Brown, 1971). Displacement of the membrane to such positive voltages always attenuated the light response to stimuli given shortly afterward (when the cell was bathed in ASW). Hence, we were careful not to pass enough current through

an NaCl electrode to allow the voltage to approach the negative resistance region of the *I–V* curve. Nonetheless, we observed a small decrement in the light response subsequent to a relatively small depolarization produced by current passed through a KCl-filled pipette. This is seen in Fig. 7 A; the effect lasted only 20–30 sec. Thus, there was no progressive decrement of the receptor potential during the iontophoretic injection of K^+, and any decrement of the receptor potential following the injection was both small and short-lived compared to that seen after Ca^{++} or Na^+ injections.

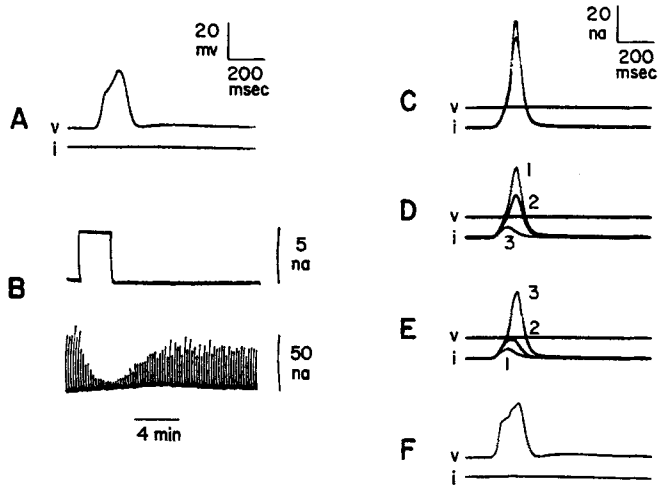

FIGURE 6. Effect of intracellular injection of Na^+ on light-induced current. Two 3 M KCl–filled electrodes and one 1 M NaCl–filled electrode were positioned inside the cell simultaneously. (A), receptor potential elicited by a brief flash. (B), lower trace is the record of light-induced clamp current. Upper trace is the record of current through the NaCl-filled pipette. (C), light-induced current before Na^+ injection (two superimposed traces). (D), light-induced currents became progressively smaller during Na^+ injection; numbers indicate order in time. (E), light-induced currents recovered after Na^+ injection; numbers indicate order in time. (F), receptor potential recorded 5 min after end of record in (B). Traces labeled *v* are membrane voltages. Traces labeled *i* are clamp currents. Positive inward membrane currents are plotted as upward deflections.

Injection of Lithium Ions

By a method analogous to the preceding experiments, we impaled a single photoreceptor cell with two micropipettes, one filled with 4 M LiCl, the other with 3 M KCl. We monitored membrane voltage with one electrode and injected constant current through the other. Thus, we could compare the effects of iontophoresis of Li^+ and K^+ into the cell. Results of such experiments show that the effects of passing Li^+ and K^+ into the cell are indistinguishable. Typical records are shown in Fig. 7 B. Prolonged injection of lithium ions did not produce a progressive decrement in the light response,

nor an after-hyperpolarization, such as that observed with the injection of sodium ions.

Light Responses in Low-Ca^{++} and High-Ca^{++} Seawaters

As previously reported by Millecchia and Mauro (1969 *a*), the receptor potential begins to become larger when calcium ions are removed from the bathing solution; however, the absence of extracellular Ca^{++} leads to an

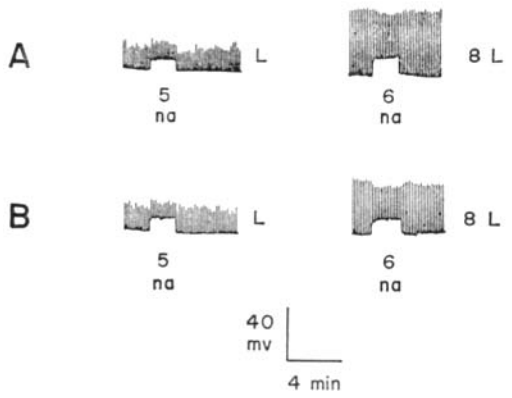

FIGURE 7

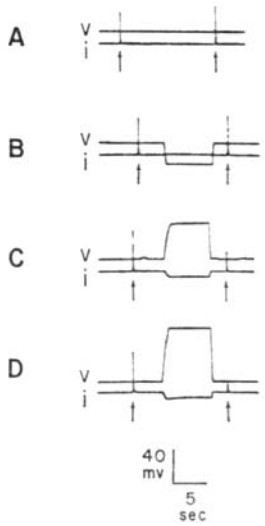

FIGURE 8

FIGURE 7. Membrane voltages recorded during intracellular iontophoretic injection of potassium or lithium ions. (A), injection of Li^+; (B), injection of K^+. In both records there is no decrement of the receptor potential during injection. There is a small, short-lived decrement following injection if stimuli are given during the injection.

FIGURE 8. Effect of displacing membrane voltage on subsequent light responses. Traces marked *v* are membrane voltages. Traces marked *i* are clamp currents. All stimuli are of fixed intensity, 50 msec duration, and are marked by vertical arrows. (A), control. (B), hyperpolarization does not affect the size of subsequent light response. (C) and (D), light response is markedly smaller following a large, positive excursion of membrane voltage.

irreversible decrease in the response to light if the treatment is longer than a few minutes. To extend the time in which the effects of a low extracellular Ca^{++} concentration could be obtained reversibly, we reduced the calcium concentration from 10 mM in ASW to 0.1 mM in our low-Ca^{++} SW. The effect of such treatment on the receptor potential is seen in Figs. 9–11. With low intensity stimuli, the transient and steady phases of the receptor potential both increased slightly in size. With high intensity light, the steady phase began to grow when the extracellular Ca^{++} was lowered, and after 1 min or

more could become very large (Fig. 11); the receptor potential became almost "square" (Fig. 10 D). With very bright stimuli, this steady phase could overshoot 0 v (Fig. 11). In voltage clamp, however, we still could obtain both transient and steady phases of light-induced current in low-Ca^{++} SW (Fig. 10 D).

With more prolonged treatment in low-Ca^{++} SW, the transient component became even less evident; the rising edge of the receptor potential became slower (Fig. 14 F). In this condition, the light-induced current also did not have a transient phase. The wave shape of the responses returned to normal when the preparation was again bathed in ASW.

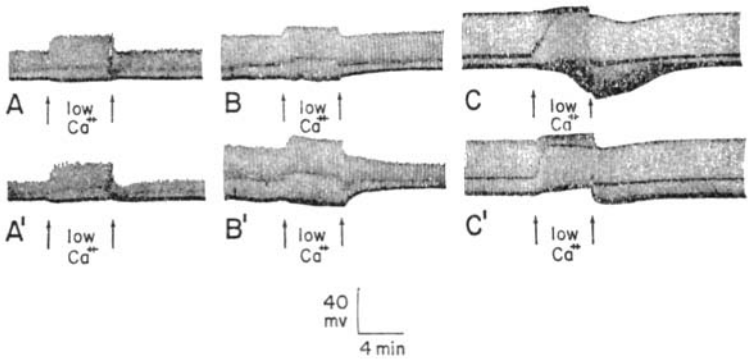

FIGURE 9. Receptor potentials recorded in low-Ca^{++} SW; two cells recorded simultaneously. (A), (A'), with low intensity stimuli, transient phase became larger when ASW was replaced by low-Ca^{++} SW in extracellular bath. (B), (B'), stimuli 16 times brighter than (A). Both transient and steady phases became larger in low-Ca^{++} SW. (C), (C'), stimuli 256 times brighter than (A). Steady phase becomes very large in low-Ca^{++} SW. All stimuli are of 0.5 sec duration. Cells were bathed in ASW except during perfusion with low-Ca^{++} SW (arrows).

Conversely, when the external calcium ion concentration is raised (to 50 mM Ca^{++}, with no sulfate present), the receptor potential and the light-induced current tend to become smaller (Fig. 12 A and C). Control responses were measured in seawater with normal Ca^{++} concentration (10 mM) with all the sulfate ions replaced by chloride ions ($SO_4^{=}$-free SW).

Injection of Sodium Ions in Low-Ca^{++} and High-Ca^{++} Seawaters

The reduction in responsiveness of the photoreceptor cell which occurs when the calcium concentration is raised either outside or inside the cell led us to ask if the decrement in light response produced by sodium injection might be mediated by a mechanism involving the calcium ions. To examine this, we injected sodium ions into photoreceptor cells bathed in low-Ca^{++} seawater (containing 0.1 M Ca^{++}). The results are shown in Fig. 13 A, B, and

C. First, a constant current of +10 na was passed through the NaCl-filled pipette for 30 sec while the preparation was bathed in ASW (10 mM Ca^{++}). The size of the receptor potential was reduced markedly during the injection and slowly recovered afterwards (Fig. 13 A). The preparation was then

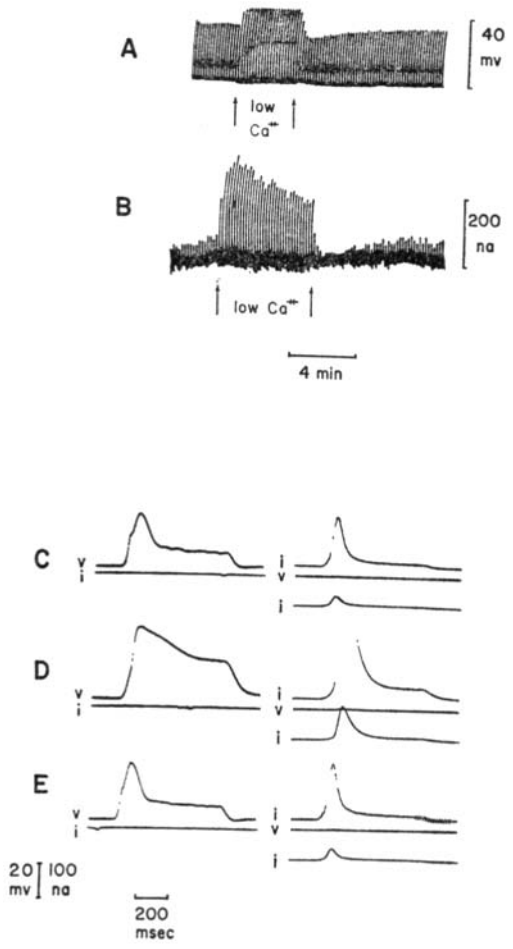

FIGURE 10. Effect of low-Ca^{++} SW on receptor potentials and light-induced currents. (A), receptor potentials elicited by 0.5 sec flashes, 1/10 sec. The cell was bathed in ASW except during perfusion with low-Ca^{++} SW (arrows). (B), light-induced current; stimuli as in (A). (C) before, (D) during, and (E) after the perfusion with low-Ca^{++} SW. Oscilloscope records of single voltage responses from (A) are shown in the left column; single light-induced current responses are shown in the right column. Traces marked *v* are membrane voltage; traces marked *i* are currents supplied by the voltage clamp (at high and low gain); inward positive membrane currents are plotted as upward displacements. Current calibration for the low gain traces = 100 na, for the high gain traces = 20 na. The oscillations in the current trace are artifacts from the perfusion system.

perfused with low-Ca^{++} seawater (Fig. 13 B); the receptor potential increased in amplitude. To insure that the receptor potential was not now at saturation amplitude, the stimuli were attenuated 16-fold; these attenuated stimuli elicited smaller responses. When we then injected sodium ions (10 na for 30 sec), no decrement in the receptor potential was seen (Fig. 13 B). The same

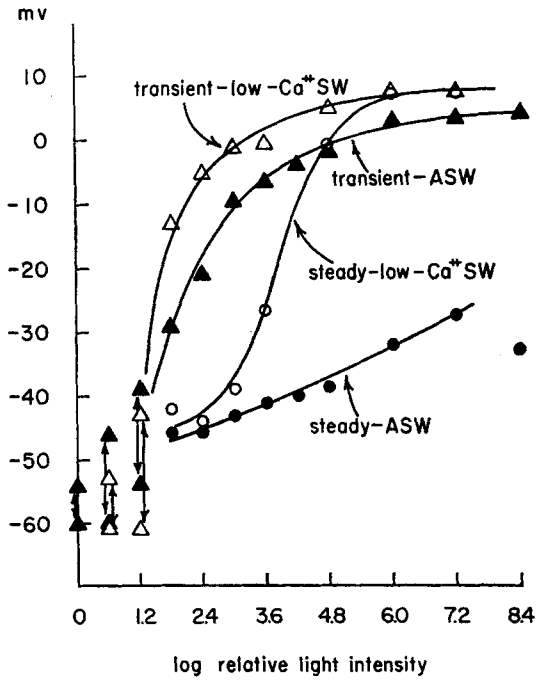

FIGURE 11. Amplitude of the receptor potential *versus* log relative light intensity. At low intensities, the light responses are "noisy" due to "quantum bumps"; the arrows indicate maximum and minimum values for the responses. In ASW, the transient phase (filled triangles) saturates at high intensities. The plateau of the receptor potential continues to grow with light intensity (except for the brightest light). In low-Ca^{++} SW, the transient phase increases more rapidly with increasing intensity, and reaches a more positive saturation voltage than in ASW (except for dim lights); the plateau also rapidly increases with intensity and saturates. At high light intensities, the receptor potential appears "square." Stimuli: 1 sec duration given 1/10 sec.

result was obtained if 100-fold dimmer flashes were used for stimuli. In this latter case, both transient and steady phases were present in the receptor potentials recorded in low-Ca^{++} SW (Fig. 10).

In contrast, the iontophoretic injection of Na^{+} into a photoreceptor cell produced a larger and more rapid diminution of the receptor potential in high-Ca^{++} SW than in normal ASW (Fig. 13 D, E, and F). The rate of diminution was greater in high-Ca^{++} SW even if stimuli 100 times brighter than those in the $SO_4^{=}$-free SW control were given (compare Figs. 13 E and F).

Thus, the magnitude of the decrement of the light response which is produced by the injection of Na^+ depends on the concentration of calcium ions in the seawater bath. At higher extracellular concentrations of Ca^{++} the rate and extent of the decrement are greater, and vice versa. In contrast,

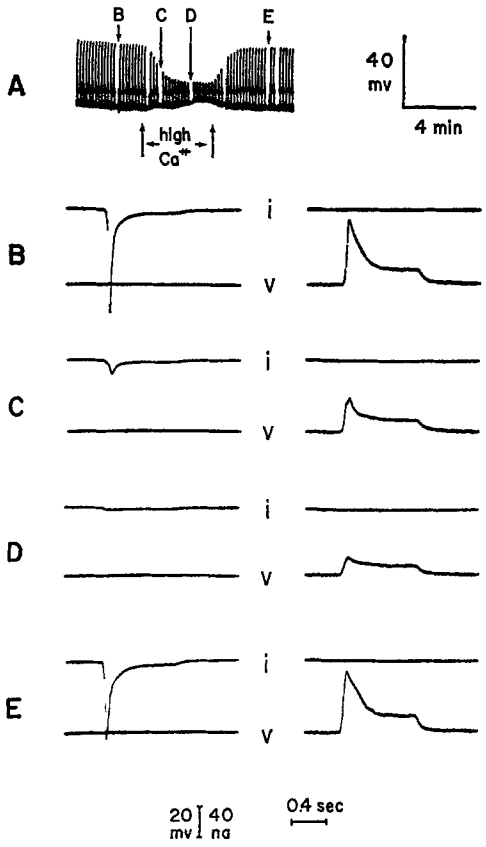

FIGURE 12. Effect of increased extracellular Ca^{++} on receptor potentials and light induced current. (A), chart record of membrane voltage. Cell was bathed in $SO_4^{=}$-free SW; lower arrows mark the interval of the high-Ca^{++} SW perfusion. Light-induced current (i) was measured at several intervals; inward positive membrane currents are plotted as upward displacements. (B)–(E), light-induced currents (left column) correspond to intervals labeled in (A). Receptor potentials, labeled v (right column), were the first responses following each interval of voltage clamp.

intracellular injection of Ca^{++} still leads to the diminution of the light response after removal of 99% of the Ca^{++} from the external bath (Fig. 14).

DISCUSSION

The size of the light response recorded from photoreceptor cells in the ventral eye of *Limulus* is progressively reduced during the intracellular injection of

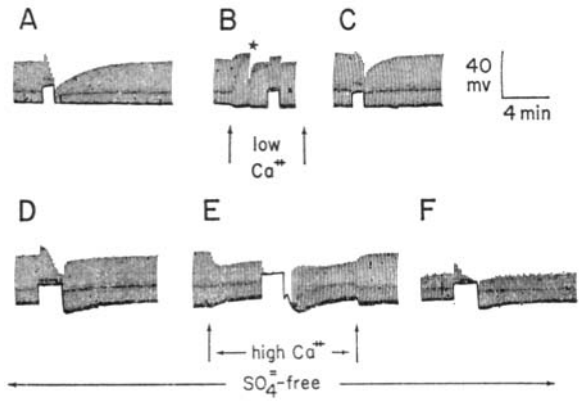

FIGURE 13. Effect of extracellular calcium concentration on intracellular Na^+ injection. All stimuli were of 0.5 sec duration. In (A), (B), and (C) +10 na was injected for 1 min through a 1 M NaCl-filled pipette. (A), Na^+ injection; the amplitude of receptor potentials became progressively smaller during injection and recovered slowly following the injection. (B), initially, the light intensity was as in (A). At the time marked by the star, the light intensity was reduced 16-fold. There is no decrement of the receptor potential during or after Na^+ injection while the cell was bathed in low-Ca^{++} SW. (C), as in (A) except light intensity was 16 times brighter. (D), (E), and (F) are from another cell bathed in $SO_4^=$-free SW, +5 na injected for 2 min through the pipette filled with 1 M NaCl. (D), Na^+ injection; stimuli 256 times brighter than (F). (E), light intensity as in (D). Vertical arrows mark period of high-Ca^{++} SW perfusion. Na^+ injection produces extreme degradation of light response. The rate of degradation is greater than that produced by the same Na^+ injection with 256 times dimmer light, in ASW. (F), Na^+ injection in $SO_4^=$-free SW.

calcium ions (Fig. 1) or sodium ions (Fig. 3), whereas the injection of the same, or a larger, amount of lithium or potassium ion (Fig. 7) does not produce this effect. The depolarization of the membrane, or the transmembrane current, itself does not produce the progressive reduction in size of the receptor potential. Moreover, a decrement in light-induced current is produced while the membrane voltage is clamped to the resting potential during and after the iontophoretic injection of Na^+ (Fig. 6) and Ca^{++} (Lisman and Brown, unpublished data). Thus, we conclude that a rise of the intracellular concentration of calcium or sodium ions attenuates the light response of ventral photoreceptor cells.

We have also shown that, following the iontophoretic injection of K^+ or Li^+, there is a relatively small and short-lived decrement of the light response, if the membrane was depolarized (to less than −10 mv) during the injection. Following comparable iontophoretic injections of Ca^{++} or Na^+, however, there is a pronounced, long-lived reduction in size of the light response which recovers slowly.

Compared to Ca^{++} injections, a larger quantity of Na^+ was required to

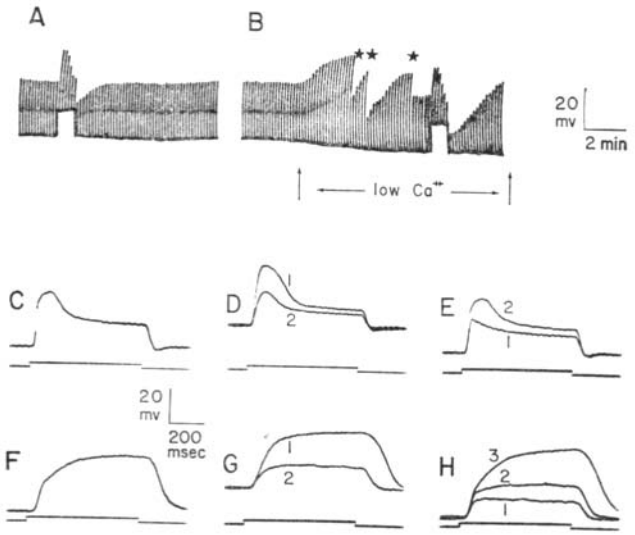

FIGURE 14. Intracellular iontophoretic injection of Ca^{++}. In both (A) and (B), Ca^{++} passed out of a Ca^{++}-EGTA electrode by +5 na for 1 min. (A), during Ca^{++} injection, there was a progressive diminution of the receptor potentials, and recovery after the injection. (B), cell was perfused with low-Ca^{++} SW (arrows). Light intensity was reduced three times (stars); the final intensity was 16 times less than the original Ca^{++} injection again produced a progressive diminution of the receptor potential. Oscilloscope records of receptor potentials for chart record (A) are seen in: (C) before Ca^{++} injections, (D) the first (*1*) and last (*2*) responses during the injection, and (E) the first (*1*) and tenth (*2*) responses after injection. For the chart record (B): (F) before Ca^{++} injection, (G) the first (*1*) and last (*2*) responses during the injection, and (H) the first (*1*) and two later (*2*, *3*) responses after the injection.

produce an equivalent decrement of the light response. We had to pass 1–10 times more current through the NaCl-filled electrode than through a Ca^{++}-EGTA–filled electrode to produce comparable decrements. The transport number of Na^+ out of a 1 M NaCl pipette is close to unity, whereas the transport number of Ca^{++} out of a Ca^{++}-EGTA pipette is approximately 0.2 (P. Brandt and J. Reuben, personal communication). Hence, we had to inject at least 5–50 times more Na^+ than Ca^{++} to produce comparable effects. Nevertheless, since calcium ions probably differ from sodium ions in their intracellular binding and diffusion, as well as in their transport out of the cell, we cannot quantify exactly the ratio of injected ions necessary to produce a comparable effect on the light response.

Millecchia and Mauro (1969 *b*) proposed that there is a light-activated Na^+-conductance increase in *Limulus* ventral photoreceptor cells. Brown and Mote (1971) have shown that, with partial replacement of Na^+ by $Tris^+$ or NaCl by sucrose, the reversal potential (V_{Rev}) of the current induced by

brief stimulin nearly follows the Nernst equation for values of $[Na^+]_{out}$ close to normal; that is, the slope of V_{Rev} *versus* log $[Na^+]_{out}$ is approximately 55 mv/10-fold change in $[Na^+]_{out}$, at 430 mM = $[Na^+]_{out}$. If sodium ions are the only charge-carrying species then we can determine if the quantity of Na^+ which we inject intracellularly is sufficient to cause a large enough shift in V_{Rev} to explain the decrement of the light response, as follows.

Millecchia and Mauro (1969 *b*) reported that the reversal voltage (V_{Rev}) of the light-induced current in a cell stimulated repetitively by a 2.5 sec flash given once very 10 sec, was approximately +20 mv; we have confirmed this (Lisman and Brown, 1971). Using the Nernst relation ($E_{Na} = V_{Rev} = RT/F \ln [Na^+]_{out}/[Na^+]_{in}$ where $[Na^+]_{in}$ and $[Na^+]_{out}$ are the intracellular and extracellular concentrations of sodium ions, respectively), we calculate that for $V_{Rev} = +20$ mv, with the extracellular concentration of Na^+ being 430 mM, the inside concentration of sodium ions, $[Na^+]_{in}$, is 190 mM. Compared to other nerve cells which generate physiological responses via sodium ion conductance increase mechanisms, this value for $[Na^+]_{in}$ seems high. In squid photoreceptors, for comparison, Adams and Hagins (1960) determined by flame photometry that $[Na^+]_{in}$ was 110 mM (giving a Nernst equilibrium voltage of +34 mv).

Using $[Na^+]_{in} = 110$ mM as a lower limit, we can compute the maximum change in V_{Rev} expected during a typical iontophoretic injection of Na^+. We can compute the upper limit of the number of sodium ions injected: for example, $(5 \times 10^{-9}$ amps) $\times$ 120 sec $\times$ 1/96,500 $\simeq 6 \times 10^{-12}$ mole of sodium ions, assuming a transport number of unity for our micropipette. The large photoreceptor cells in the ventral eye have been found to be spindle-shaped cells, measuring 50–100 $\times$ 150–200 μ (Stell and Ravitz, 1970). Hence, assuming that a cell has a volume of at least 3×10^{-10} liter, if no ions were transported out of the cell, the largest increase in internal concentration of Na^+ could be 20 mM. Therefore, if our original assumption of a single charge-carrying species (i.e., Na^+) is correct, we find that the Nernst equilibrium potential should become less than 5 mv more negative due to the injection of Na^+. In agreement with this calculation, we measure less than 5 mv change in the reversal potential of the light response, both during and after Na^+ injection. This rules out the possibility that the large reduction of the amplitude of the light response which occurs with typical injections of Na^+ is due to forcing the reversal voltage to be so negative that it nearly reaches the dark resting voltage. In other words, the Nernst potential for sodium ions is relatively unchanged; if the simple conductance increase hypothesis is correct, the injection of Na^+ leads to a reduction in the magnitude of the light-induced conductance increase.

Our data suggest that the decrease in the light-induced conductance change, caused by an increase in intracellular Na^+, is mediated by Ca^{++}.

The extent of the decrement in the light response produced by the intracellular injection of sodium ions depends on the concentration of calcium ions in the seawater bath; the higher the extracellular Ca^{++} concentration, the greater and more rapid the diminution in the light response produced by Na^+ injection. With a very low extracellular concentration of Ca^{++}, virtually no decrement is seen during and after Na^+ injection (Fig. 13). Moreover, as is described in a companion paper (Brown and Lisman, 1972), any procedure which ought to decrease the activity of the sodium pump, allowing the accumulation of intracellular Na^+, leads to a decrease in the size of the light response, but only if there is a significant concentration of calcium ions in the external bath. We also find that an increase in intracellular Ca^{++} concentration by direct injection (Fig. 1) causes a reduction in the size of the light response. Therefore, we propose that the intracellular injection of Na^+ leads to an increase in intracellular Ca^{++} concentration, and that raising intracellular Ca^{++} concentration by any means reduces the responsiveness of the cell to light.

That an increase in $[Ca^{++}]_{in}$ can be caused by an increase in $[Na^+]_{in}$ has been proposed in other systems. For example, a sodium-calcium exchange mechanism has been postulated for squid giant axon (Baker, 1970).

We have shown that an increase in intracellular Ca^{++} leads to a decrease in the light-induced conductance. This effect of Ca^{++} may explain the wave shape of the response to long-duration stimuli. The light-induced current has an initial, large transient, followed by a smaller steady value. If intracellular Ca^{++} concentration rises during the light response, then the diminution of current occurring after the transient might be caused by the increase in $[Ca^{++}]_{in}$.

One mechanism by which intracellular Ca^{++} might rise during the light response is as follows. There is a rise in intracellular Na^+ due to the influx of sodium ions during the light response; this rise in $[Na^+]_{in}$ would lead to a rise in $[Ca^{++}]_{in}$ as proposed above. However, a sodium-stimulated Ca^{++} entry cannot account entirely for the wave shape of the light response; when the membrane voltage is clamped more positive than reversal voltage for the rapid, light-induced changes in current (e.g., +40 mv), the net current carried by Na^+ ought to be outward. Hence, under this condition, there would be no influx of Na^+ to stimulate Ca^{++} entry. However, the light response still has a transient phase–steady phase wave shape (Millecchia and Mauro, 1969 *b*; Lisman and Brown, 1971).

Alternatively, the calcium ion concentration would rise during the light response if there were a light-induced increase in Ca^{++} conductance; calcium ions would flow into the cell down their electrochemical gradient. In the barnacle photoreceptor (Brown et al., 1970), the reversal potential of the light response strongly depends on $[Ca^{++}]_{out}$, in sodium-free seawater.

This suggests that a light-induced Ca^{++} conductance change may exist in the barnacle. At present we have no evidence which can exclude the possibility that a small part of the light-induced current is carried by Ca^{++} in *Limulus* ventral photoreceptors.

We thank Doctors F. Baumann, A. L. F. Gorman, W. Loewenstein, K. J. Muller, and J. F. Nolte for their helpful discussions. We also thank Doctors P. Brandt and J. Reuben for their suggestion of, and assistance with, the Ca^{++}-EGTA electrodes.

This work was supported by National Institutes of Health Grants EY-00834, EY-00835, and EY-00897.

Received for publication 15 November 1971.

REFERENCES

ADAMS, R. G., and W. A. HAGINS. 1960. The ionic composition of squid photoreceptors. *Biol. Bull.* **119**:300.

BAKER, P. F. 1970. Na-Ca exchange agross the nerve cell membrane. *In* Calcium and Cellular Function. A. W. Cuthbert, editor. The Macmillan Company, New York. 96.

BROWN, J. E., and J. E. LISMAN. 1972. An electrogenic sodium pump in *Limulus* ventral photoreceptor cells. *J. Gen. Physiol.* **59**:720.

BROWN, J. E., and M. I. MOTE. 1971. Na^{+}-dependence of reversal potential of light-induced current in *Limulus* ventral photoreceptors. *Biol. Bull.* **141**:379.

BROWN, H. M., S. HAGIWARA, A. KOIKE, and R. MEECH. 1970. Membrane properties of a barnacle photoreceptor examined by the voltage-clamp technique. *J. Physiol. (London).* **208**:385.

CLARK, A. W., R. MILLECCHIA, and A. MAURO. 1969. The ventral photoreceptor cells of *Limulus*. I. The microanatomy. *J. Gen. Physiol.* **54**:289.

DEMOLL, R. 1914. Die Augen von *Limulus*. *Zool. Jahrb. Abt. Anat. Ontog. Tiere.* **38**:443.

FUORTES, M. G. F. 1958. Electric activity of cells in the eye of *Limulus*. *Amer. J. Ophthalmol.* **46**:210.

KIKUCHI, R., K. NAITO, and I. TANAKA. 1962. Effects of sodium and potassium ions on the electrical activity of single cells in the lateral eye of the horseshoe crab. *J. Physiol. (London).* **161**:319.

LISMAN, J. E., and J. E. BROWN. 1971. Two light-induced processes in the photoreceptor cells of *Limulus* ventral eye. *J. Gen. Physiol.* **58**:544.

MILLECCHIA, R., and A. MAURO. 1969 *a*. The ventral photoreceptor cells of *Limulus*. II. The basic photoresponse. *J. Gen. Physiol.* **54**:310.

MILLECCHIA, R., and A. MAURO. 1969 *b*. The ventral photoreceptor cells of *Limulus*. III. A voltage-clamp study. *J. Gen. Physiol.* **54**:331.

STELL, W. K., and M. J. RAVITZ. 1970. The structure of neurons in the ventral photoreceptor organ of the horseshoe crab, *Limulus polyphemus*. *J. Cell Biol.* **47** (2, pt. 2):202 *a*.

TASAKI, K., Y. TSUKAHARA, S. HO, M. J. WAYNER, and W. Y. YU. 1968. A simple, direct and rapid method for filling micropipettes. *Physiol. Behav.* **3**:1009.

TOMITA, T. 1956. The nature of action potentials in the lateral eye of the horseshoe crab as revealed by simultaneous intra- and extracellular recording. *Jap. J. Physiol.* **6**:327.