

Effects of Intracellular Injection of Calcium Buffers on Light Adaptation in *Limulus* Ventral Photoreceptors

J. E. LISMAN and J. E. BROWN

From the Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138, the Department of Anatomy, Vanderbilt University School of Medicine, Nashville, Tennessee 37232, and the Marine Biological Laboratories, Woods Hole, Massachusetts 02543. Dr. Lisman's present address is the Department of Biology, Brandeis University, Waltham, Massachusetts 02154.

ABSTRACT The calcium sequestering agent, EGTA, was injected into *Limulus* ventral photoreceptors. Before injection, the inward membrane current induced by a long stimulus had a large initial transient which declined to a smaller plateau. Iontophoretic injection of EGTA tended to prevent the decline from transient to plateau. Before injection the plateau response was a nonlinear function of light intensity. After EGTA injection the response-intensity curves tended to become linear. Before injection, bright lights lowered the sensitivity as determined with subsequent test flashes. EGTA injection decreased the light-induced changes in sensitivity. Ca-EGTA buffers having different levels of free calcium were pressure-injected into ventral photoreceptors; the higher the level of free calcium, the lower the sensitivity measured after injection. The effects of iontophoretic injection of EGTA were not mimicked by injection or similar amounts of sulfate and the effects of pressure injection of EGTA buffer solutions were not mimicked by injection of similar volumes of pH buffer or mannitol. The data are consistent with the hypothesis that light adaptation is mediated by a rise of the intracellular free calcium concentration.

INTRODUCTION

The photoreceptors of *Limulus* exhibit the property of adaptation (Hartline and McDonald, 1947; Benolken, 1962; Fein and DeVoe, 1973; Srebro and Behbehani, 1974; Lisman and Brown, 1975). Sensitivity is reduced when the cell is exposed to light (light adaptation). After the termination of an adapting stimulus, the sensitivity gradually recovers (dark adapts) to its original value. The experiments described in this paper bear upon the mechanism of light and dark adaptation.

The receptor potentials recorded from *Limulus* ventral photoreceptors are positive going (depolarizing). These voltage changes are due to a light-induced increase in the membrane conductance; the consequent light-

induced current can be measured by a voltage clamp technique and is carried mainly by an influx of sodium ions (Millecchia and Mauro, 1969 *a, b*; Brown and Mote, 1974). Using a voltage clamp to measure light responses, both the response induced by a brief flash and the initial response to a step change of light are approximately proportional to the change in illumination (over a limited range) (Lisman and Brown, 1975); the proportionality constants, both of which depend on the history of illumination, have different units. Although we have no mathematically rigorous relationship between the two proportionality constants, both reflect the underlying responsiveness of the conductance-increase mechanism. In this paper the term " $\Delta g/\text{intensity}$ " will be used to describe the responsiveness of the conductance-increase mechanism.

Several lines of evidence indicate that in *Limulus* photoreceptors $\Delta g/\text{intensity}$ can decrease without a significant reduction in the concentration of rhodopsin. For example, sensitivity can be altered by a brief flash whose intensity is no more than 100 times brighter than that necessary to evoke a single discrete wave (Lisman and Brown, 1975). Such stimuli isomerize only a tiny fraction of the billion rhodopsin molecules in a single cell (Lisman and Bering, 1973). Furthermore, analysis of the noisiness of the responses to dim and moderate intensity stimuli in *Limulus* lateral eye shows that the probability of an incident photon affecting membrane conductance is not strongly influenced by an adapting stimulus (Dodge et al., 1968). Finally, after the photopigment is maximally stimulated, it returns to its original state long before the cell reaches its minimum threshold (Fein and DeVoe, 1973; Fein and Cone, 1973). Thus, the photoreceptors must have a mechanism for regulating the conductance increase per absorbed photon that is independent of the rhodopsin cycle.

Lisman and Brown (1972 *a*) have proposed that light causes an increase in the intracellular free calcium concentration, Ca_i , which in turn mediates changes of $\Delta g/\text{intensity}$ (light adaptation). This hypothesis is supported by the observation that increasing Ca_i by iontophoretic injection reversibly decreases the sensitivity of the cell (Lisman and Brown, 1972 *b*; Brown and Lisman, 1974). Also, Brown and Blinks (1974) have shown directly, using aequorin, that light induces a rise in Ca_i . In a companion paper (Lisman and Brown, 1975) we argue that three different aspects of the responses to light are interrelated consequences of changes in the $\Delta g/\text{intensity}$. First, the responses to test flashes are reduced during and after a bright light, i.e., the cell is light adapted. Second, a prolonged adapting stimulus elicits a large initial transient response which declines to a plateau. Third, the plateau response is normally a nonlinear function of intensity. Our analysis suggests that light-induced changes of intracellular free calcium mediate changes of the $\Delta g/\text{intensity}$. If light-induced changes of intracellular free calcium were

reduced then changes of the Δg /intensity would be reduced; consequently, adaptational changes would be reduced, there would be less decline from transient to plateau, and the plateau current would be a more linear function of intensity.

In the present experiments we have sought to stabilize Ca_i by intracellular injection of calcium buffers (Portzehl et al., 1964). A preliminary abstract of some of the results has been published previously (Lisman and Brown, 1971 *a*).

METHODS

The dissection of the ventral rudimentary eye of *Limulus*, apparatus for recording membrane voltage, and the voltage clamp circuitry are described elsewhere (Lisman and Brown, 1971 *b*). Tungsten light sources were used in most of the experiments. In a few, (e.g. Fig. 2) a Strobonar 880 (Honeywell, Inc., Denver, Colo.) was used.

Cells were bathed in artificial seawater (Brown and Lisman, 1972). The microelectrode used for passing current was filled with 3 M KCl. Voltage measurements were made with the microelectrode used for iontophoretic or pressure injection. Microelectrodes were filled using the glass fiber technique of Tasaki et al. (1968). When filled with 3 M KCl, the electrodes had resistances between 4 and 10 m Ω .

Injection solutions designed to buffer Ca_i were prepared with the sequestering agent, EGTA, which selectively binds calcium over magnesium by about five orders of magnitude (Caldwell, 1970). The solution used for iontophoretic injection of EGTA was 0.3 M EGTA brought to pH 7.8 with KOH. The EGTA was negatively charged and could be injected into the cytoplasm by iontophoresis. Injection currents hyperpolarized the cell no more than 30 mV.

The solutions used for pressure injection of EGTA were buffered to pH 7.0 with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid). The compositions of the calcium ion buffer solutions and other pressure injection solutions are given in Table I. Microelectrodes were selected to optimize the injection of buffer solution when pressure was applied, while restricting the leakage of solution when none was

TABLE I
INJECTION SOLUTIONS

Solution	Ca(OH) ₂	EGTA	HEPES	KOH*	Na ₂ ³⁵ SO ₄	H ₂ ³⁵ SO ₄ ‡	Mannitol	KCl
	<i>M</i>	<i>M</i>	<i>M</i>		<i>M</i>	<i>M</i>	<i>M</i>	<i>M</i>
<i>a</i>	—	0.1	0.01	*	0.029	—	—	—
<i>b</i>	—	0.5	0.05	*	—	0.0001	—	—
<i>c</i>	0.084	0.1	0.01	*	0.029	—	—	—
<i>d</i>	0.420	0.5	0.05	*	0.029	—	—	—
<i>e</i>	0.49	0.5	0.05	*	—	0.0001	—	—
<i>f</i>	—	—	0.01	*	0.029	—	0.1	0.1
<i>g</i>	—	—	0.04	*	—	0.0001	0.4	0.4
<i>h</i>	—	0.1	0.5	*	—	0.0001	—	—
<i>i</i>	—	—	0.05	*	—	—	—	—

* KOH added to titrate to pH 7.0.

‡ Carrier-free H₂³⁵SO₄ added, about 10⁻⁴ M.

applied. We nevertheless often observed some effect of intracellular EGTA before the application of pressure. A buffer solution was injected by applying a low pressure (5–15 psi) to the back of a microelectrode for several minutes. In some experiments we determined the total volume of the solution injected by including radioactive $^{35}\text{SO}_4$ in the injection solutions. After the injection, the radioactivity in the ventral eye was determined by partially digesting the tissue in Pronase (Calbiochem, San Diego, Calif.) (20 mg/ml at room temperature), adding Aquasol (New England Nuclear, Boston, Mass.), and counting in a liquid scintillation counter. The volume of solution injected was calculated from the specific activity of the injection solution and the measured radioactivity of the tissue.

The experiments reported in this paper were performed on cells which met two criteria: (a) the plateau phase of the receptor potential evoked by a bright stimulus was 10 mV or greater, (b) the responses measured by the two intracellular microelectrodes were identical. Many cells did not produce large quantum bumps in the dark-adapted state.

Neutral density filters were calibrated using a spectrophotometer. Light intensity was measured with a PIN-10 photodiode (United Detector Technology, Santa Monica, Calif.) in the unbiased mode. Intensities were measured after the beam passed through infrared cutoff filters. White light was used in most experiments, but in some a narrow band interference filter (530 nm) was placed in the beam. The conditions of illumination for each experiment are given in each figure legend. Because the light beam passed through a nerve bundle before reaching the photoreceptor, and because the beam was not always well focused on the cell, the actual light intensity at the cell surface was lower than the intensities given by 0.05–0.55 log units (see Lisman and Brown, 1975). All experiments were carried out at room temperature (22–24°C).

RESULTS

Although there are several membrane processes that can contribute to light-dependent membrane currents in a ventral photoreceptor (Millecchia and Mauro, 1969 *b*; Lisman and Brown, 1971 *b*; Brown and Lisman, 1972), only the conductance increase mechanism (called the “fast process” by Lisman and Brown, 1971 *b*) contributes significantly to the current when the cell is voltage clamped to resting potential. In the experiments reported here, the clamping voltage was always resting potential, and thus the light-activated currents were nearly proportional to the light-activated conductance. In the figures, positive inward membrane current is plotted downward.

Effect of EGTA Injections on the Response Waveform

Responses to a range of stimulus intensities were examined before and after iontophoretic EGTA injection. At very low light intensities EGTA injection decreased the amplitudes of the responses (Fig. 1). Decreases also occurred after pressure injection of EGTA (Fig. 4 B).

The response to a long bright stimulus in a normal cell was characterized

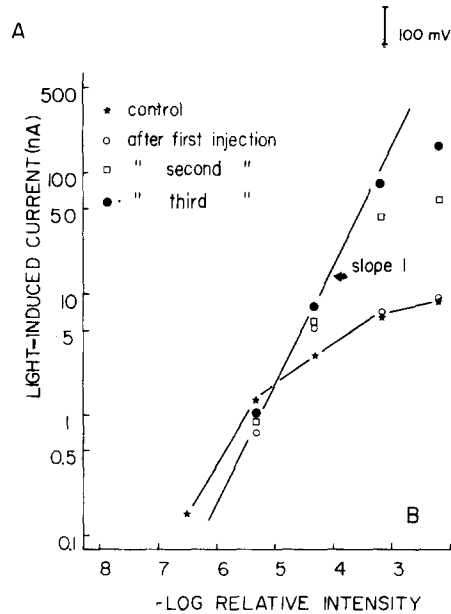
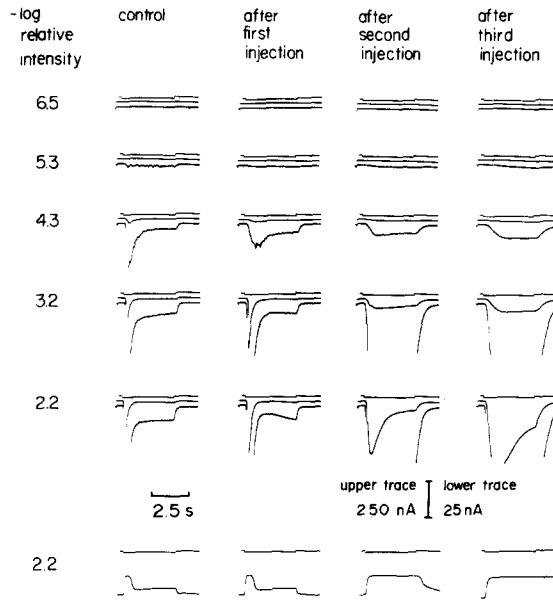


FIGURE 1. The effect of EGTA injection on the response waveform and on response-intensity curves. Measurements were made after the iontophoretic injection of increasing amounts of EGTA. The first injection was 1.8×10^{-6} C, the second was 4.3×10^{-6} C, the third was 3.6×10^{-6} C. (A) Light-induced currents were simultaneously recorded at two different gains. Currents were measured at five light intensities: $-\log$ relative intensity is given in left column. Voltage responses were measured at only one intensity (bottom of figure). The intensity of the unattenuated beam was 4×10^{-4} W/cm² (monochromatic, 530 nm). The top trace in each set of traces is the output of a light monitor. Before injection, the (control) responses to stimuli brighter than -5.3 had two phases, the transient and plateau. Injection of EGTA tended to reduce the ratio of transient current to plateau current. (B) Response-intensity curves were constructed from the data in A. The currents plotted were measured at the end of the plateau response. Before EGTA injection, the response-intensity curve was nonlinear. The greater the amount of EGTA injected, the closer the points approached a line of slope 1 (representing a linear relationship between current and intensity).

by a large initial transient phase which declined to a smaller plateau phase. The ratio of transient to plateau current was large, often greater than 30 to 1. The effect of EGTA injection was to reduce the ratio of transient current to plateau current, thus making the response more nearly square. This occurred both because of an increase in the plateau response and because of a reduction in the amplitude of the transient (see responses at -3.2 in Fig. 1 A). Such square responses are not due simply to slowing of the transient phase of the response, since they also could be elicited by stimuli 10 s long.

The effect of EGTA injection on the response waveform depended on the amount of EGTA injected. For example, the first injection in Fig. 1 affected the responses to dim lights but had relatively little effect on the responses to bright lights. The second injection produced little further effect on the response to dim lights, but produced dramatic changes in the responses to brighter lights; the response at -3.2 still had a large transient-to-plateau ratio after the first injection but became square after the second injection. The third injection produced little further effect at this intensity, but tended to make the response to still brighter stimuli (-2.2) more square. Therefore, it would appear that the brighter the stimulus the higher the intracellular EGTA concentration required to prevent the decline from transient to plateau.

Voltage responses to bright stimuli before and after EGTA injections are shown at the bottom of Fig. 1 A. After the second injection the voltage waveform became nearly square even though the membrane currents measured at resting potential were not. The maximum value of the membrane potential during the light response was $+10$ to $+15$ mV, which is approximately the reversal voltage for the conductance-increase mechanism (Millecchia and Mauro, 1969 *b*; Lisman and Brown, 1971 *b*). We surmise that both the transient and plateau currents were sufficient to bring the voltage close to reversal potential.

The relationship between the amplitude of the plateau current and stimulus intensity was affected by EGTA injection. In the normal cell the plateau current was a linear function of intensity if the stimuli were dim, and became nonlinear at brighter intensities (see control curve in Fig. 1 B). After EGTA injection (Fig. 1 B) the response-intensity curve became nearly linear over a larger range of intensities.

Effect of EGTA Injection on Light Adaptation

To study light adaptation before and after EGTA injection, we examined the effects of long stimuli on the responses to subsequent test flashes (Fig. 2). The energy of these test flashes was chosen to be in the linear portion of the response versus energy curve (Lisman and Brown, 1975). Before injection of EGTA, an intensity for the prolonged stimulus was found such that it evoked

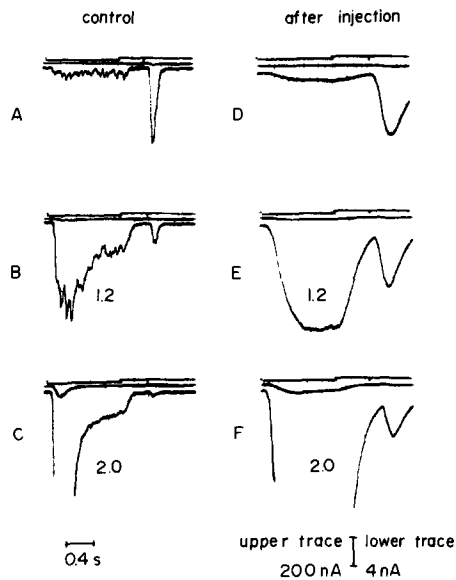


FIGURE 2. The effect of EGTA injection on light adaptation. The effect of long stimuli on the responses to brief test flashes was observed before (control) and after the iontophoretic injection of EGTA into the cell (6.7×10^{-6} C). Light-induced currents were recorded at two different gains. The uppermost trace in each set is the output of a light monitor. A, B, and C were recorded before injection. The log intensity of the long stimulus relative to that in A is given beside traces B and C. In A, the intensity of the long stimulus was 3.7×10^{-10} W/cm². The brighter the long stimulus, the greater the reduction in sensitivity as measured by the test flashes. D, E, and F were recorded after EGTA injection. The intensity of the long stimulus in D was increased to 2.2×10^{-9} W/cm² to induce about the same plateau current as in A. The log intensity of the long stimulus relative to that in D is given beside traces E and F. The energy of the test flashes in D, E, and F was 1.5 log units brighter than in A, B, and C. After injection, the brighter prolonged stimuli produced comparatively little reduction in sensitivity as measured by the test flashes. Both long and short stimuli were monochromatic (530 nm).

a small maintained current and had little or no effect on the response to a subsequent test flash (Fig. 2 A). Dimmer long stimuli also had no effect. Long stimuli of brighter intensities attenuated responses to the test flashes (Fig. 2 B and C). Since the response versus energy relationship was linear for the test stimuli, the proportional reduction in response amplitude was equal to the reduction in sensitivity. After injection of EGTA, an energy of the test flash was again chosen to be in the linear range. The intensity of the long stimulus was adjusted to elicit the same maintained current as was measured in Fig. 2 A. When the intensity of the prolonged stimulus was raised, the same relative increases in intensity produced much less attenuation of the responses to subsequent test flashes than before injection. We conclude that EGTA injection tended to prevent light-induced changes in sensitivity.

Effect of EGTA Injection on the Kinetics of Excitation

EGTA injection decreased the rate of rise of the current at the onset of the response to a stimulus (Fig. 1 A); the falling edge of the response was similarly affected. The duration of the responses to brief flashes became longer after EGTA injection (Fig. 2).

The responses in *Limulus* have been interpreted as the summation of discrete waves (Rushton, 1961; Dodge et al., 1968), each of which arises from the isomerization of a single rhodopsin molecule (Fuortes and Yeandle, 1964). In this framework, the prolongation of the responses to brief flashes could occur either because of a broadening of the latency distribution of the discrete waves, or because of a prolongation of the discrete waves themselves. The responses to long, dim stimuli became less noisy after EGTA injection (Fig. 1 A). This would not be expected if only the latency distribution of the discrete waves was affected by EGTA injection and suggests that the intracellular EGTA causes a lengthening of the discrete waves.

Controls for Iontophoretic Injection of EGTA

Iontophoretic injections of sulfate ions equal in magnitude to the largest iontophoretic EGTA injections altered neither the decline from transient to plateau nor the response versus intensity curves. Experiments in which radioactive sulfate was included in the injection solution indicate that the membrane of *Limulus* photoreceptors is not freely permeable to sulfate. Since the average charge on EGTA is -2 at physiological pH, EGTA and sulfate should have equivalent effects on the osmolarity and ionic strength of the cytoplasm. Thus, neither hyperpolarization of the membrane during injection nor changes in the ionic or osmotic strength of the cytoplasm arising from iontophoretic EGTA injection were the cause of the observed effects.

It might be argued that the effect of EGTA injection arises from a chemical reaction between EGTA and some membrane component. This possibility is difficult to rule out; however, iontophoretic injections of EGTA had little or no effect on resting potential or on steady-state current-voltage curves measured in the dark. Thus at least some components of the membrane were not substantially altered by EGTA injection.

Injection of EGTA Buffers Having Various Free Calcium Concentrations

EGTA buffers having various free calcium concentrations were pressure injected into ventral photoreceptors. The value of the free calcium concentration maintained by the buffers in the cytoplasm cannot be accurately calculated (see Discussion), however, the more Ca added to the EGTA solution, the higher the concentration of free calcium.

The pressure injection of Ca-EGTA buffers produced alterations in the

response waveform similar to those observed after the iontophoretic or pressure injection of EGTA alone; the responses to dim stimuli were attenuated, whereas the plateau responses to bright stimuli became larger and the responses tended to become square (Fig. 3). The effects of the injection of a Ca-EGTA buffer depended on the level of free calcium in two ways. First, the higher the free calcium concentration, the higher the intensity required to induce a small current of chosen amplitude (i.e. higher threshold; Table II). Second, after injection of a Ca-EGTA buffer, the plateau response tended to be a linear function of stimulus intensity. Thus, the graphs of the plateau response versus intensity made before and after injection have a single intersection (Fig. 4) (referred to as the "crossover" intensity). The intensity at which the intersection occurred depended on the level of free calcium in the injected buffer; the higher the level of free calcium, the higher the light intensity at the intersection.

The effects of iontophoretic or pressure injection of EGTA were partly reversible. In some instances we observed that responses which were nearly square after EGTA injection, recovered a larger transient-to-plateau ratio over a 30-min period after the injection.

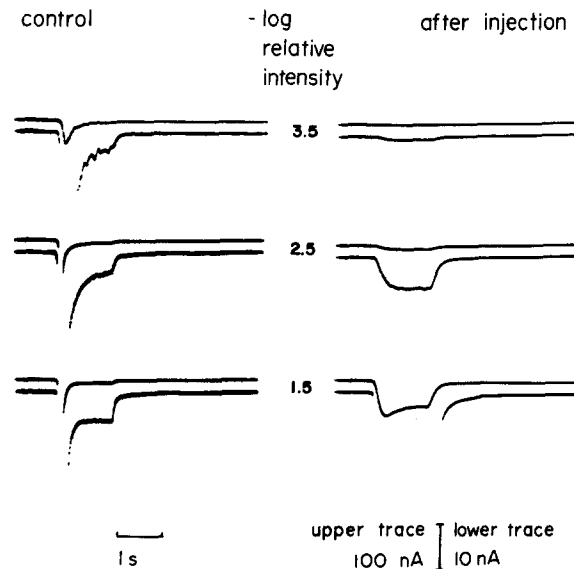


FIGURE 3. Light-induced currents before (left column) and after (right column) the injection of Ca^{++} -EGTA solution of composition $\text{Ca}(\text{OH})_2/\text{EGTA} = 0.84$. Pairs of traces were recorded simultaneously at two different gains. Numbers on the center column indicate $-\log$ relative light intensities. White light was used in these experiments. The absolute intensity of the unattenuated beam (\log relative intensity = 0) was not measured at the time of the experiments but is estimated to have been between 10^{-4} and 3×10^{-3} W/cm².

TABLE II
EFFECT OF INJECTING Ca-EGTA BUFFERS HAVING
DIFFERENT FREE CALCIUM CONCENTRATIONS

	Injection solution	Volume injected (10 ⁻¹⁰ liters)	Crossover intensity (-log ₁₀ relative intensity)*	Threshold after injection (-log ₁₀ relative intensity)*
EGTA, no Ca added	<i>a</i>	0.08	3.50	3.60
	<i>a</i>	0.08	3.70	4.80
	<i>b</i>	0.12	3.20	4.20
	<i>b</i>	0.16	3.40	4.30
	<i>a</i>	0.16	3.90	4.30
	<i>a</i>	0.17	3.50	4.90
	<i>a</i>	0.27	3.40	4.20
	<i>h</i>	0.27	3.65	4.20
	<i>h</i>	0.30	3.40	4.20
	<i>a</i>	0.31	3.50	4.20
	<i>h</i>	0.45	3.75	4.60
	<i>a</i>	0.49	3.80	4.45
	<i>a</i>	1.00	3.40	4.05
		Mean±SD	3.55±0.20	4.31±0.33
Ca(OH) ₂ /EGTA = 0.84	<i>d</i>	0.13	3.40	3.80
	<i>c</i>	0.17	2.50	3.10
	<i>c</i>	0.27	3.00	3.35
	<i>d</i>	0.31	2.60	3.50
	<i>d</i>	0.59	3.05	3.85
	<i>c</i>	0.68	2.70	4.60
	<i>c</i>	1.39	2.40	3.40
	<i>c</i>	1.49	2.90	3.40
	<i>c</i>	1.74	2.50	3.45
		Mean±SD	2.78±0.33	3.61±0.44
Ca(OH) ₂ /EGTA = 0.98	<i>e</i>	0.08	1.65	3.00
	<i>e</i>	0.22	1.80	3.10
	<i>e</i>	0.25	1.35	2.40
	<i>e</i>	0.29	2.10	3.60
	<i>e</i>	0.32	2.35	3.00
	<i>e</i>	0.39	0.80	2.20
	<i>e</i>	0.40	1.70	4.10
	<i>e</i>	0.44	0.95	3.40
	<i>e</i>	0.44	2.45	2.80
	<i>e</i>	1.06	1.65	2.80
	Mean±SD	1.68±0.54	3.04±0.56	

* White light was used in these experiments. The absolute intensity at $-\log_{10}$ relative intensity = 0 was not measured at the time of the experiments but is estimated to be between 10^{-4} and 3×10^{-3} W/cm²

Injection of Other Substances

To determine if the effects of pressure injection of EGTA solutions were due to an artifact of the pressure injection procedure, we substituted (mole for mole) mannitol for EGTA and used a ³⁵SO₄ marker to measure the volume

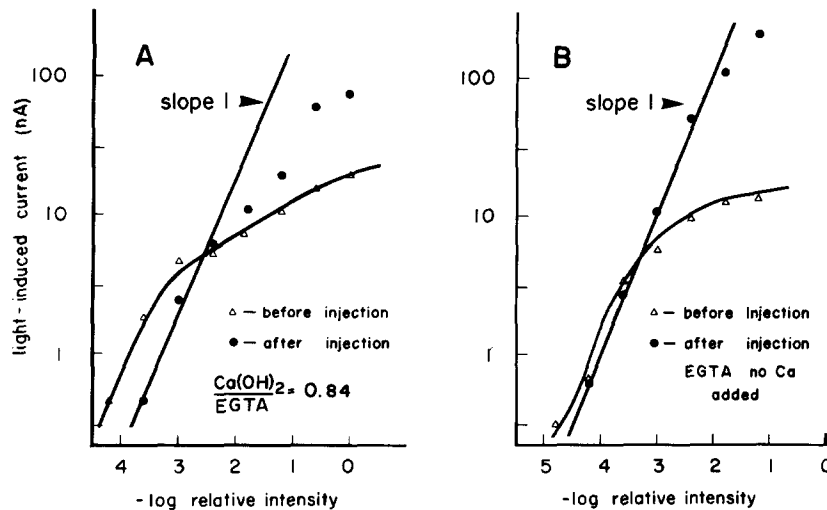


FIGURE 4. Light-induced plateau current as a function of stimulus intensity, before and after pressure injection of calcium buffers. (A) Before (triangles) and after (filled circles) injection of Ca^{++} -EGTA buffer ($\text{Ca}(\text{OH})_2/\text{EGTA} = 0.84$). At crossover, $-\log$ relative intensity is 2.6. (B) Before (triangles) and after (filled circles) injection of EGTA (no Ca^{++} added) solution. At crossover, $-\log$ relative intensity is 3.3. White light was used in these experiments. The absolute intensity of the unattenuated beam (\log relative intensity = 0) was not measured at the time of the experiments but is estimated to have been between 10^{-4} and 3×10^{-3} W/cm².

of fluid injected. Table III summarizes the results of experiments on 15 cells. The volume of ventral photoreceptors is as large as about 5×10^{-10} liters (Clark et al., 1969; Stell and Ravitz, 1970). When the injection volume was a large fraction of the photoreceptor volume (about 2×10^{-10} liters), the injection of mannitol solutions sometimes produced effects similar to those seen after smaller injections of EGTA solutions: the slope of the response-intensity curve became closer to unity for bright stimuli. In addition, the response-intensity curves measured before and after injection crossed. However, in order to produce a slope of the response-intensity curve similar to that produced by injection of about 0.3×10^{-10} liters of an EGTA buffer, it was necessary to inject more than 7×10^{-10} liters of the mannitol solutions. After these large mannitol injections, the cells were visibly swollen; often a bubble protruded from the cell. Swelling was not observed after typical injections of EGTA solutions. We conclude that the stabilization of $\Delta g/\text{intensity}$ produced by pressure injection of EGTA was not an artifact of the injection process, and we suspect that the stabilization which occurred after massive injection of mannitol solutions was fundamentally different from that which occurred after injections of smaller volumes of EGTA solutions.

Since injections of small volumes of mannitol or HEPES solutions some-

TABLE III
MANNITOL INJECTIONS

Injected solution (Table I)	Injected volume (10^{-10} liters)	Crossover ($-\log_{10}$ relative intensity)*	Effect on response vs. intensity curve
<i>f</i>	0.03	—	Depress
<i>f</i>	0.05	—	No change
<i>f</i>	0.40	—	No change
<i>g</i>	0.44	—	Depress
<i>g</i>	0.93	—	Depress
<i>f</i>	1.25	—	Depress
<i>f</i>	1.34	—	Depress
<i>g</i>	1.95	3.0	Crossover
<i>f</i>	2.03	—	Depress
<i>g</i>	3.00	0.9	Crossover
<i>g</i>	3.19	—	No change
<i>f</i>	4.10	2.5	Crossover
<i>f</i>	4.44	—	Depress
<i>f</i>	7.84	1.9	Crossover
<i>g</i>	12.06	2.9	Crossover

* White light was used in these experiments. The absolute intensity at $-\log_{10}$ relative intensity = 0 was not measured at the time of the experiments but is estimated to be between 10^{-4} and 3×10^{-3} W/cm².

times cause a depression of the response at all intensities, we cannot exclude the possibility that the small reduction in response to dim lights caused by pressure injection of an EGTA (no Ca added) solution (Fig. 4 B) is artifactual. However, since the threshold measured after injection depended on the concentration of calcium in the EGTA buffer injected, it is highly unlikely that the rise in threshold caused by buffer injection was wholly an artifact of the pressure injection procedure.

Both the mannitol solutions and EGTA solutions contained a pH buffer (HEPES, $pK = 7.55$), and EGTA itself buffers pH. To test if the changes produced by EGTA injection were simply due to the buffering of pH, we injected 0.5 M HEPES adjusted to pH 7.0 with KOH. For injections as large as 0.9×10^{-10} liters (intracellular buffer concentration approximately 100 mM), we observed either no change in the response-intensity curve, or a decrease in the response amplitude at all intensities. We conclude that the buffering of the hydrogen ion concentration was not responsible for the effects of the injection of Ca-EGTA buffers. Another possibility is that a light-induced rise in Ca_i causes a release of protons from EGTA and that this reduction of pH tends to stabilize Δg /intensity. This seemed not to be the case, since responses became square even when the injection solution contained a five times greater concentration of pH buffer than EGTA (solution *h* in Tables I and II).

DISCUSSION

In *Limulus* photoreceptors the responsiveness of the conductance increase mechanism ($\Delta g/\text{intensity}$) is normally not constant but is time dependent and a function of stimulus intensity (Lisman and Brown, 1975). Our results show that the $\Delta g/\text{intensity}$ tends to be stabilized by the intracellular injection of Ca-EGTA buffers. This is evident in three ways: EGTA injection tends (a) to prevent the decline from transient to plateau during a prolonged stimulus, (b) to cause the plateau response to become a linear function of intensity, and (c) to prevent illumination from attenuating responses to subsequent test flashes. The controls suggest that these effects of EGTA injection are not artifacts of the injection process. Assuming that the effects of EGTA are due to its calcium-buffering action, we conclude that changes in the concentration of intracellular free calcium normally mediate light-induced changes of the $\Delta g/\text{intensity}$. This conclusion is further strengthened by the observation (Brown and Blinks, 1974) of a light-induced rise in Ca_i (detected directly with the luminescent protein, aequorin), which can be prevented by intracellular injection of EGTA. Moreover, the amount of EGTA needed to prevent the stimulus-induced aequorin luminescence is approximately equal to that required to produce a square receptor potential.

Brown and Blinks (1974) also found that the light-induced rise in Ca_i is graded with stimulus intensity. It therefore seems plausible that more intracellular EGTA buffer is needed to stabilize the $\Delta g/\text{intensity}$ at high intensities than at low intensities (Fig. 1) because the challenge to the buffer is greater at high intensities. If the stimulus is made very bright, a decline from transient to plateau can always be observed. For this reason we can say only that EGTA injection *tends* to stabilize the $\Delta g/\text{intensity}$.

The approximate concentration of EGTA buffer in the cytoplasm after pressure injection of buffer can be computed from data in Tables I and II and a value for the cell volume of 5×10^{-10} liters (Clark et al., 1969; Stell and Ravitz, 1970). The computed final concentration of intracellular EGTA (no calcium added) ranges between 1.6 and 45 mM. An upper limit for the concentration of EGTA after iontophoretic injection is 100 mM, if it is assumed that all the current was carried by EGTA. By comparison, an intracellular EGTA concentration of 1–10 mM is required for partial inhibition of muscle contraction (Ashley, 1967).

The ventral photoreceptors of *Limulus* have large axons, up to 20 μM in diameter (Clark et al., 1969). Diffusion of EGTA down the axon would lower the EGTA concentration in the soma and this might account for at least part of the recovery of the transient-to-plateau ratio observed after injection. The effect of EGTA injection on the light-evoked responses of an

Aplysia giant neuron also has been found to be reversible (Brown and Brown, 1973).

The value at which the Δg /intensity tends to be stabilized depends on the composition of the injected Ca-EGTA buffer. The higher the free calcium concentration in the buffer, the higher is the intensity necessary to produce a conductance change of criterion size (Table II.). This finding agrees with the earlier observation that iontophoretic injection of calcium ions reduced the responses elicited by stimuli of a given intensity (Lisman and Brown, 1972 *b*).

From the present data we can approximate the steady-state values of Ca_i required to reduce the Δg /intensity if pH_i , Mg_i , and the binding constants of EGTA are known. We assume that pH_i is 7.0 and that Mg_i is close to the value of 2 mM found in squid axon (Baker and Crawford, 1972). The binding constants for EGTA are not universally agreed upon; we use those cited by Caldwell (1970). Given these values, the solution $Ca(OH)_2$ /EGTA = 0.84 maintains a free calcium concentration of 1.2 μM ; the solution $Ca(OH)_2$ /EGTA = 0.98 maintains a concentration of 10.4 μM . These calculations indicate that raising Ca_i to levels between 1 and 10 μM can produce graded reductions in the Δg /intensity.

If Ca_i is the only factor controlling the responsiveness of the conductance-increase mechanism in the normal photoreceptor, then the crossover intensities found by injecting Ca-EGTA buffers can be used to infer that illumination normally leads to a graded increase in Ca_i . The responses elicited by dim stimuli are attenuated by injection of a Ca-EGTA buffer. We infer that an intracellular Ca-EGTA buffer provides a higher Ca_i to the binding sites controlling the Δg /intensity than would normally occur when the cell was dimly illuminated. However, relative to an uninjected cell, the plateau responses elicited by bright stimuli are larger after injection of a Ca-EGTA buffer. We infer that in the uninjected cells, the Ca_i would normally have risen (at least transiently) to a value greater than that maintained by the Ca-EGTA buffer. Similarly, we presume that when the injected buffer supplies approximately the same amount of calcium to the binding sites controlling the Δg /intensity as does the normally occurring rise in Ca_i , then the graphs of plateau response vs. intensity made before and after injection intersect. At successively higher values of free Ca_i maintained by the buffers, the crossover occurs at successively brighter stimulus intensities. Although there is variation in crossover intensity after injection of a given solution, there is little overlap in the distributions of crossover intensities (Table II). These findings are indirect evidence that light induces a rise of Ca_i which is graded with stimulus intensity, and are in agreement with the more direct evidence for a light-induced rise of Ca_i , graded with stimulus intensity, as detected with aequorin (Brown and Blinks, 1974).

When EGTA (to which no calcium was added) is injected into the photo-

receptor, the responses at low light intensities are slightly attenuated (Figs. 1, 4 B). This attenuation may be, in part, an artifact of the injection process, especially when EGTA is injected by pressure. Also the level of Ca_i maintained by the EGTA buffer may be higher than Ca_i in the normal dark-adapted cell. This might occur because of calcium contamination from the EGTA itself, the electrode glass, and the glass fibers used for filling the micro-electrode, all of which would raise the free calcium concentration of the buffer solution. Alternatively the injection of EGTA might cause the release of calcium from some intracellular store, which in turn would raise the free calcium concentration maintained by the EGTA buffer.

Fuortes and Hodgkin (1964) showed that an adapting light not only desensitized *Limulus* lateral eye photoreceptors, but also shortened the "time scale" of responses. We have shown that the pressure injection of Ca-EGTA buffers produces a desensitization of the cell that is graded with the level of free Ca in the buffer (Table II). However, as illustrated in Fig. 3, the responses can have a slower rise time after injection than before injection. A similar slowing can also be observed after the pressure injection of mannitol. Thus there appear to be factors other than Ca_i that can influence the time scale (see also Coles and Brown, 1974). This however should not be taken to exclude the possibility that both time scale and sensitivity are controlled by Ca_i during the normal functioning of the cell. We have recently studied the effect of raising Ca_i by iontophoresis and found that Ca_i injection causes both a reduction in sensitivity and a shortening of time scale (Brown and Lisman, 1975).

The results reported here suggest that a rise in Ca_i is a step mediating light-induced decreases in sensitivity. The results of Brown and Blinks (1974) indicate that there are light-induced changes in Ca_i . If the aequorin signal accurately reflects changes in Ca_i , then during a long light stimulus Ca_i increases and then declines to a less elevated level. During the same interval, the sensitivity decreases to a lower, steady level. These results suggest that (under nonsteady-state conditions) there cannot be a simple inverse proportionality between Ca_i and $\Delta g/\text{intensity}$. This difficulty of relating the time-course of Ca_i to the function presumed to be regulated by Ca_i is not a problem unique to this preparation. During prolonged depolarization of barnacle muscle, tension rises smoothly, but Ca_i rises and then falls (Ashley and Ridgeway, 1970). For barnacle muscle, the problem can be resolved (Ashley and Moiescu, 1972) if it is assumed that the sites which regulate tension bind calcium tightly. Calcium would be released so slowly from such sites that they would behave like a leaky integrator. Under these circumstances, the amount of calcium bound to the sites could correlate with tension even though Ca_i did not. The calcium controlled sites that regulate sensitivity in *Limulus* photoreceptors may have similar properties.

There is other evidence that changes in Ca_i are important in photoreceptor function. However, the exact role of calcium apparently differs in different photoreceptors. Brown and Brown (1973) have shown that a rise in Ca_i is probably the cause of the light-evoked increase in the potassium permeability observed in a light-sensitive giant neuron of the *Aplysia* abdominal ganglion. Yoshikami and Hagins (1971) have postulated that in vertebrate rods and cones, a rise in Ca_i causes a decrease in the sodium conductance of the plasma membrane, thereby generating the receptor potential. In these two cases, calcium seems to be involved mainly in the sequence of events leading to excitation (i.e., in producing a conductance change across the plasma membrane). The evidence presented in this paper and elsewhere suggests that in *Limulus*, changes in Ca_i are involved in controlling the level of adaptation.

The proposed models for *Limulus* and vertebrate photoreceptors have one important feature in common. In both a rise in Ca_i modulates the "sodium" conductance. The difference is that in vertebrate photoreceptors an increase of Ca_i is thought to decrease sodium conductance that is high in the dark. In *Limulus* the evidence suggests that an increase of Ca_i attenuates the light-activated sodium conductance. We wish to stress, however, that nothing in our data indicates that calcium interacts directly with the permeability pathway (presumably a channel or carrier). The transduction events which couple the isomerization of rhodopsin to an increase in membrane conductance are not understood. If indeed calcium is involved in regulating the responsiveness of the conductance-increase mechanism calcium could act at any stage of the transduction process.

We wish to thank Drs. P. K. Brown, J. A. Coles, G. Fain, A. Fein, A. E. Stuart, and G. Wald for their helpful discussions. We wish to thank especially Dr. K. J. Muller for his help in developing the pressure-injection technique and for many fruitful discussions.

This work was supported by NIH grants EY-00834 (to J. E. B.), EY-00835 (to J. E. B.), EY-00508 (to Dr. G. Wald), and EY-01496 (to J. E. L.), and by a Grass Foundation Fellowship to J. E. L.

Received for publication 8 January 1975.

REFERENCES

- ASHLEY, C. C. 1967. The role of cell calcium in the contraction of single cannulated muscle fibers. *Am. Zool.* 7:647.
- ASHLEY, C. C., and D. G. MOISESCU. 1972. Model for the action of calcium in muscle. *Nat. New Biol.* 237:208.
- ASHLEY, C. C., and E. B. RIDGWAY. 1970. On the relationships between membrane potential, calcium transient and tension in single barnacle muscle fibres. *J. Physiol. (Lond.)* 209:105.
- BAKER, P. F., and A. C. CRAWFORD. 1972. Mobility and transport of magnesium in squid giant axons. *J. Physiol. (Lond.)* 227:855.
- BENOLKEN, R. M. 1962. Effects of light and dark adaptation processes on the generator potential of the *Limulus* eye. *Vis. Res.* 2:103.

- BROWN, A. M., and H. M. BROWN. 1973. Light response of a giant *Aplysia* neuron. *J. Gen. Physiol.* **62**:239.
- BROWN, J. E., and J. R. BLINKS. 1974. Changes in intracellular free calcium during illumination of invertebrate photoreceptors: detection with aequorin. *J. Gen. Physiol.* **64**:643.
- 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 J. E. LISMAN. 1974. Light-adaptation is mimicked by intracellular Ca^{++} injection in *Limulus* ventral photoreceptors. *Fed. Proc.* **33**:434.
- BROWN, J. E., and J. E. LISMAN. 1975. Intracellular Ca modulates both sensitivity and time scale in *Limulus* ventral photoreceptors. Submitted to *Nature*.
- BROWN, J. E., and M. I. MOTE. 1974. Ionic dependence of reversal voltage of the light response in *Limulus* ventral photoreceptors. *J. Gen. Physiol.* **63**:337.
- CALDWELL, P. C. 1970. Calcium chelation and buffers. In *Calcium and Cellular Function*. Cuthbert, editor. St. Martins, London. 10-16.
- CLARK, A. W., R. MILLECCHIA, and A. MAURO. 1969. The ventral photoreceptor cells of *Limulus*. I. The microanatomy. *J. Gen. Physiol.* **54**:289.
- COLES, J. A., and J. E. BROWN. 1974. Effects of increased intracellular pH-buffering capacity on the light-induced response of *Limulus* ventral photoreceptors. *Biol. Bull. (Woods Hole)*. **147**:473.
- DODGE, F., B. KNIGHT, and J. TOYODA. 1968. Voltage noise in *Limulus* visual cells. *Science (Wash. D. C.)*. **160**:88.
- FEIN, A., and R. CONE. 1973. *Limulus* rhodopsin: rapid return of transient intermediates to the thermally stable state. *Science (Wash. D. C.)*. **182**:495.
- FEIN, A., and R. P. DEVOE. 1973. Adaptation in the ventral eye of *Limulus* is functionally independent of the photochemical cycle, membrane potential and membrane resistance. *J. Gen. Physiol.* **61**:273.
- FUORTES, M. G. F., and A. L. HODGKIN. 1964. Changes in time scale and sensitivity in ommatidia of *Limulus*. *J. Physiol. (Lond.)*. **172**:239.
- FUORTES, M. G. F., and S. YEANDLE. 1964. Probability of occurrence of discrete potential waves in the eye of *Limulus*. *J. Gen. Physiol.* **47**:443.
- HARTLINE, H. K., and P. R. McDONALD. 1947. Light and dark-adaptation of single photoreceptor elements in the eye of *Limulus*. *J. Cell. Comp. Physiol.* **30**:225.
- LISMAN, J. E., and H. BERING. 1973. Electrophysiological measurement of the number of rhodopsin molecules in *Limulus* ventral photoreceptor cells. Society for Neuroscience Annual Meeting Abstracts. **3**:357.
- LISMAN, J. E., and J. E. BROWN. 1971 *a*. Intracellular injection of Ca^{++} -EGTA solutions in *Limulus* ventral photoreceptors. *Biol. Bull. (Woods Hole)*. **141**:395.
- LISMAN, J. E., and J. E. BROWN. 1971 *b*. Two light-induced processes in the photoreceptor cells in *Limulus* ventral eye. *J. Gen. Physiol.* **58**:544.
- LISMAN, J. E., and J. E. BROWN. 1972 *a*. The effects of intracellular Ca^{2+} on the light response and on light adaptation in *Limulus* ventral photoreceptors. In *The Visual System: Neurophysiology, Biophysics and their Clinical Applications*, G. B. Arden, editor. Plenum Publishing Co., New York. 23-33.
- LISMAN, J. E., and J. E. BROWN. 1972 *b*. The effects of intracellular iontophoretic injection of calcium and sodium ions on the light response of *Limulus* ventral photoreceptors. *J. Gen. Physiol.* **59**:701.
- LISMAN, J. E., and J. B. BROWN. 1975. Light-induced changes of sensitivity in *Limulus* ventral photoreceptors. *J. Gen. Physiol.* **66**:473.
- MILLECCHIA, R., and A. MAURO. 1969 *a*. The ventral photoreceptor cells of *Limulus*. II. The basic photoreponse. *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.
- PORTZEHL, H., P. C. CALDWELL, and J. C. RUEGG. 1964. The dependence of contraction and

- relaxation of muscle fibres from the crab *Maia squinado* on the internal concentration of free calcium ions. *Biochim. Biophys. Acta.* **79**:581.
- RUSHTON, W. A. H. 1961. The intensity factor in vision. *In* Light and Life. W. D. McElroy and B. Glass, editors. The Johns Hopkins University Press. 706-723.
- SREBRO, R., and M. BEHBEHANI. 1974. Light adaptation in the ventral photoreceptor of *Limulus*. *J. Gen. Physiol.* **64**:166.
- 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**:202a.
- TASAKI, K. Y., S. TSUKAHARA, M. J. HO, M. J. WAYNER, and W. Y. YU. 1968. A simple, direct and rapid method for filling micropipettes. *Physiol. Behav.* **3**:1009.
- YOSHIKAMI, S., and W. A. HAGINS. 1971. Ionic basis of dark current and photocurrent of retinal rods. *Biophys. Soc. Annu. Meet. Abstr.* **11**:47a.