Relationship Between Light Sensitivity and Intracellular Free Ca Concentration in *Limulus* Ventral Photoreceptors

A Quantitative Study Using Ca-selective Microelectrodes

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ABSTRACT The possible role of Ca ions in mediating the drop in sensitivity associated with light adaptation in *Limulus* ventral photoreceptors was assessed by simultaneously measuring the sensitivity to light and the intracellular free Ca concentration (Ca_i); the latter was measured by using Ca-selective microelectrodes. In dark-adapted photoreceptors, the mean resting Ca_i was 3.5 ± 2.5 μM SD ($n = 31$). No correlation was found between resting Ca_i and absolute sensitivity from cell to cell. Typically, photoreceptors are not uniformly sensitive to light; the Ca_i rise evoked by uniform illumination was 20–40 times larger and faster in the most sensitive region of the cell (the rhabdomeral lobe) than it was away from it. In response to a brief flash, the Ca_i rise was barely detectable when 10^2 photons were absorbed, and it was saturated when $\sim 10^5$ photons were absorbed. During maintained illumination, starting near the threshold of light adaptation, steady Ca_i increases were associated with steady desensitizations over several log units of light intensity: a 100-fold desensitization was associated with a 2.5-fold increase in Ca_i. After a bright flash, sensitivity and Ca_i recovered with different time courses: the cell was still desensitized by ~0.5 log units when Ca_i had already recovered to the prestimulus level, which suggests that under those conditions Ca_i is not the rate-limiting step of dark adaptation. Ionophoretic injection of EGTA markedly decreased the light-induced Ca_i rise and increased the time to peak of the light response, but did not alter the resting Ca_i, which suggests that the time to peak is affected by a change in the capacity to bind Ca^{2+} and not by resting Ca_i. Lowering the extracellular Ca^{2+} concentration (Ca_o) first decreased Ca_i and increased sensitivity. Longer exposure to low Ca_o resulted in a further decrease of Ca_i but decreased rather than increased

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sensitivity, which suggests that under certain conditions it is possible to uncouple Ca_i and sensitivity.

INTRODUCTION

Absorption of light by the visual pigment leads to conductance changes in the membrane of all photoreceptors. In most invertebrate photoreceptors, the response to light consists of a graded depolarization that is mainly the result of an increase in sodium conductance (Fulpius and Baumann, 1969; Millecchia and Mauro, 1969*a*, *b*; H. M. Brown et al., 1970). Background illumination decreases both the sensitivity to light and the time scale of the photoresponse, the two main aspects of light adaptation.

Because of their large somas (Clark et al., 1969), the ventral photoreceptors of *Limulus* have been widely used for studying the molecular mechanisms underlying light adaptation (Millecchia and Mauro, 1969*a*, *b*; J. E. Brown and Lisman, 1975; Lisman and Brown, 1975*b*; Fein and Lisman, 1975; Fein and Charlton, 1977*a*, 1978*a*). Consequently, Lisman and Brown (1972*a*) proposed that a light-induced increase in intracellular free Ca concentration (Ca_i) is a factor controlling light adaptation. J. E. Brown and Blinks (1974), using aequorin, and J. E. Brown et al. (1977), using arsenazo III, have shown that light causes a transient increase in Ca_i. J. E. Brown and Blinks (1974) also showed that the light-induced Ca_i increase was graded with light intensity. The light-induced desensitization is also graded with intensity (Lisman and Brown, 1975*a*), which supports the idea that the two might be related. However, because of difficulties inherent in these photometric methods (Blinks, 1978; Blinks et al., 1982), it has not been possible to demonstrate that the light-induced changes in receptor sensitivity and Ca_i are in fact quantitatively related.

As an alternative to these photometric methods, we have used Ca-selective microelectrodes to measure Ca_i. Their main advantage is that they allow the quantification of both resting Ca_i and stimulus-induced changes in Ca_i. In addition, Ca-selective microelectrodes based on the neutral carrier ligand ETH 1001 (Oehme et al., 1976) are fairly insensitive to variations of physiological concentrations of Na^+, K^+, H^+, and Mg^{2+}.

Our intention was to address the following questions: (*a*) Is the light-induced Ca_i increase confined to the region of light absorption or does Ca_i increase throughout the cell? (*b*) Are Ca_i and sensitivity correlated during different degrees of light adaptation? (*c*) After illumination, during the time course of dark adaptation, are sensitivity and Ca_i correlated? (*d*) What determines the amount of Ca_i increase per absorbed photon? Is it the intensity of the light stimulus, the adaptational state of the cell, or the value of the resting Ca_i? (*e*) Are the changes in sensitivity induced by injection of Ca chelators and by variations of Ca_o mediated by a change in Ca_i?

Our results indicate that the measured Ca_i rise, evoked by a uniform illumination, depends on the region of the cell in which it is recorded and is typically 20–40 times larger and faster in the most sensitive region of the photoreceptor than it is away from it. During light adaptation, there is a direct relationship between Ca_i and sensitivity: we show that light induces a graded steady increase in Ca_i that is associated over several log units of background intensity with a

graded steady desensitization. The relationship is such that a 100-fold reduction in sensitivity is associated with a 2.5-fold steady increase in Ca_i. We examined the uniqueness of this relationship in three ways: (*a*) by following the kinetics of recovery of both Ca_i and sensitivity (after a bright flash) during dark adaptation, (*b*) by impeding the normal light-induced Ca_i increase by injecting a Ca chelator, and (*c*) by altering the resting Ca_i by bathing the photoreceptors in low external free Ca concentration.

Preliminary reports of some of these findings have already appeared (Levy, 1982; Levy and Fein, 1982, 1983).

METHODS

Preparation and Perfusion System

The preparation is the same as that first described by Millecchia and Mauro (1969*a*). During experiments, the ventral nerve was superfused with artificial seawater (ASW; see Table I) at a rate of 0.6 ml/min. For the low-Ca_o experiments, two different solutions were used; in one (0.1-Ca ASW), the free Ca concentration was lowered to 0.1 mM and NaCl was increased to 460 mM; in the other (0-Ca ASW), 2 mM EGTA was added in place of the Ca salt and NaCl was increased to 460 mM. The level of the bath (volume 0.2 ml) above the nerve was kept low in order to reduce the Ca electrode stray capacitance (see Cornwall and Thomas, 1981) and to provide good visualization of the electrode tip. The level of the bath was empirically found to be low enough when the meniscus formed by the Ca electrode and the bath could be seen under the light microscope while the cell to be impaled was in focus (magnification 400; see Fig. 1 for an example). Experiments were done at 20–23°C.

Electrical Arrangement

The Ca microelectrode potential (E_{Ca}) was recorded by a custom-built high-input impedance electrometer (Pelagic Electronics, Falmouth, MA), which was based on the AD 515L operational amplifier (Analog Devices, Norwood, MA) and had capacity neutralization. The input bias current of the AD 515L was checked regularly by means of a 10^{12}-Ω resistor and was typically 7–10 fA. A separate intracellular voltage electrode (V electrode) was used to measure the cell's membrane potential (E_m) and to inject current, using a constant-current circuit (Fein and Charlton, 1977*b*). The potential of the V electrode was subtracted from that of the Ca microelectrode to give the Ca signal, $E_{Ca} - E_m$. Thus, the Ca signal is a measure of the intracellular free Ca concentration. E_{Ca}, E_m, and the Ca signal were displayed on a digital voltmeter and an oscilloscope, and recorded continuously on a multichannel recorder (Brush 2400, Gould Inc., Cleveland, OH). The Ca signal recorded and shown on all the figures was filtered by a 5-Hz low-pass filter.

The Ca microelectrode was held by a 5-cm-diam loudspeaker used as a jolting device. The cells were impaled with the Ca microelectrodes by applying brief voltage pulses to the loudspeaker. The bath electrode was an Ag-AgCl pellet connected to the bath via a glass capillary filled with 3 M KCl (W-P Instruments, Inc., New Haven, CT). The V electrodes were filled with 3 M KCl and had resistances of 10–30 MΩ. EGTA injection electrodes were filled with 0.3 M EGTA brought to pH 7.8 with KOH and had resistances of ~100 MΩ.

Light Stimulation

Light from a 12-V, 100-W quartz halogen lamp was used to form two beams, which were combined by a beam-splitter and focused onto the preparation through a condenser (10×,

0.25-N.A. microscope objective), according to a design by E. F. MacNichol, Jr. (details are given in Fein and Charlton, 1975). Test flashes for the measurement of sensitivity were delivered by one beam at a frequency of 0.1 Hz and adapting stimuli were delivered by the other beam. The intensity of the unattenuated white light I_o arriving onto the photoreceptor from either beam was 0.10 W/cm^2 (infrared filtered), as measured with a calibrated photometer (United Detector Technology, Santa Monica, CA). Intensities are expressed as $\log_{10} I/I_o$.

PHYSIOLOGICAL CALIBRATION OF THE LIGHT SOURCE Assuming that one discrete wave is the result of one effectively absorbed photon (Yeandle and Spiegler, 1973), one can calibrate the stimulus energy as the number of effective photons per second by counting the number of light-induced discrete waves at very low light intensities. Discrete waves were counted for numerous periods of dark and light; the discrete wave rate resulting from spontaneous activity or stray light was subtracted from the light-induced one. It was found that either beam (unattenuated) delivered $\sim 2 \times 10^8$ effective photons/s to the photoreceptors, a value close to that (1×10^8) previously found by Fein and Charlton (1977*c*).

Threshold is expressed as the intensity (I/I_o) of a 20-ms test flash that elicited an average criterion response of 4–5 mV in amplitude. Sensitivity is defined here as the reciprocal of the threshold.

Light Sensitivity Profile of the Photoreceptors

To study the spatial localization of the Ca_i increase, the sensitivity profile of the cell was determined by scanning the photoreceptor with a microspot of light. The microspot was made by a pinhole mounted on a calibrated *x-y* positioner in the light path of one beam. The diameter of the microspot focused on the photoreceptors was ~10 μm (Fig. 1). The procedure for determining the sensitivity profile of the photoreceptors was as follows. After the microspot was positioned at one end of the cell, the cell was impaled with the V electrode and allowed to dark-adapt. It was then scanned by moving the pinhole in steps of 14 μm and by presenting 20-ms-long microspot flashes whose intensities were varied to elicit a criterion response of 4–5 mV. When the threshold response amplitude was too variable, a uniform background light was presented to slightly light-adapt the cell; microspot flashes superposed on the background light were then used to scan the cell.

Although presenting such a uniform light causes a reduction in sensitivity that is not necessarily uniform, this procedure was adequate since the intent was to localize only those areas where the sensitivities were extreme. In cells where the intent was to measure Ca signals away from the most sensitive area, the scanning was usually repeated at the end of the experiment before the Ca microelectrode and the V electrode were withdrawn. When the intent was to measure Ca signals in the most sensitive area, the cell was impaled in that area with the Ca microelectrode; the magnitude and kinetics of the light-induced Ca signal increase confirmed the location of the Ca electrode.

Sometimes, by carefully orienting the photoreceptors on the side of the nerve, it was possible to better focus the microspot and to see what appeared to be two lobes (Calman and Chamberlain, 1982; Stern et al., 1982). Fig. 1*A* shows a photomicrograph of such a cell as seen through the light microscope. Fig. 1*B* shows the sensitivity profile of that cell after it had been impaled with the V electrode and tested with the microspot. It can be seen that one end of the photoreceptor is much more sensitive than the other. The sensitivity does not fall to zero away from the most sensitive area, which may be because of light scatter (Stern et al., 1982). It might be argued that what looks like two lobes could very well be a two-cell cluster. However, the sensitivity profile of the cell does not support that possibility: clusters of photoreceptors have been shown to be joined at their rhabdomeral lobes (Calman and Chamberlain, 1982). In this case, the maximum sensitivity should

be at the junction and not localized at one end of the cell, as shown by the sensitivity profile.

Area of Response to Test Flashes

For EGTA injection experiments and for some experiments in 0-Ca ASW, the area of response to test flashes was measured and a proportionality rule was applied to calculate

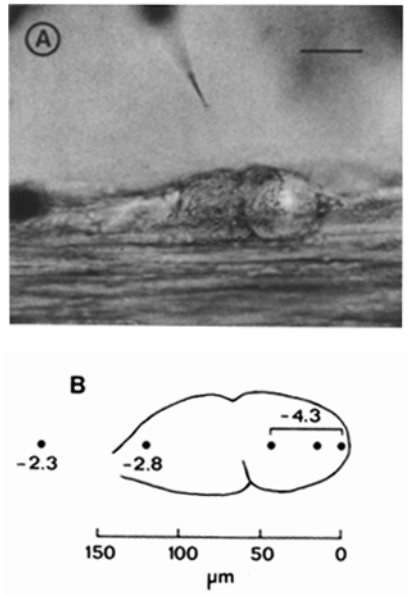

FIGURE 1. (*A*) Photomicrograph of a *Limulus* ventral photoreceptor. The nerve was pinned down in the chamber so as to orient the photoreceptor on the side of the nerve. The cell appears to have two lobes, which was confirmed by measuring the sensitivity profile of the cell with a microspot of light. The microspot can be seen in the center of the right-hand lobe. Above the photoreceptor is the Ca microelectrode, which forms a meniscus with the surface of the bath. The very tip of the Ca electrode is below the plane of the cell and is therefore out of focus. The meniscus formed by the voltage electrode and the bath is seen at the left. The tip of the voltage electrode is inside the cell. Calibration bar, 50 μm. (*B*) Light-sensitivity profile of the cell in *A* obtained by recording the intracellular voltage and scanning the cell with a microspot. Each dot corresponds to the position of the microspot. The associated numbers give the log intensity of a 20-ms test flash, which elicited a 4–5-mV criterion response: the right lobe had a much higher sensitivity. The last number on the left shows the extent of the light scatter: the test flash was given outside of the cell, but still elicited a light response.

the corrected sensitivity, based on the change in area. For the proportionality rule, we assumed that the larger the area of the criterion response was, the greater the sensitivity would be. The legend of Table VI gives the equation used to calculate the corrected sensitivity.

Measurement of Ca_i with Ca-selective Microelectrodes

Ca-selective microelectrodes were of the type in which a column of sensor, selective to ionized Ca, is contained in the open tip of a glass micropipette. We decided to use separate

single-barreled Ca and voltage microelectrodes instead of double-barreled Ca microelectrodes, because single-barreled Ca microelectrodes have better slopes between pCa 5 and 7 (pCa = $-\log[Ca^{2+}]$) and are easier to make (for a comparison between the two types of Ca electrodes, see Levy, 1979). However, there is less certainty that two separate microelectrodes will measure the same resting membrane potential.

CONSTRUCTION OF THE Ca MICROELECTRODES Construction essentially followed the method of Tsien and Rink (1980, 1981). In brief, microelectrodes were made from cleaned, thin-walled capillaries, silanized in an oven at 220°C, and slightly beveled (see Werblin, 1975) to ~0.3 μm across the bevel. The pipettes were then back-filled with a Ca-buffered solution of pCa ~6.5 (mM: 5 $CaCl_2$, 10 EGTA, 100 KCl, 10 K·BES, pH 7.0); suction applied to the back of the pipette was used in order to fill the tip with a 30–80-μm-long sensor column. Such electrodes had resistances of 3–10 × 10^{11} Ω (details are given in Levy, 1983).

The polyvinyl chloride (PVC)-gelled sensor used in all experiments had the following composition: 9–11% neutral carrier ligand (21192, Fluka, Hauppauge, NY), 2.5–4% tetraphenylarsonium tetrakis (*p*-biphenylyl) borate (IHS, W-P Instruments, Inc.), 13–16% PVC (kindly provided by Dr. R. Y. Tsien, University of California, Berkeley, CA), and 2-nitro-phenyl octyl ether (73732 Fluka). This mixture was dissolved in ~2.5 times its weight in tetrahydrofuran (THF; 18,656-2, Aldrich Chemical Co., Milwaukee, WI). It was noticed, in accordance with Tsien and Rink (1981), that viscosity is the most important element in making good Ca microelectrodes: the more viscous the sensor, the lower the detection limit for Ca^{2+}. Therefore, before use, THF was allowed to evaporate until the sensor reached an acceptable viscosity.

Macro PVC electrodes were made by gluing, with a drop of THF, a flat PVC membrane containing the neutral carrier ligand onto a 2-mm-o.d. PVC tubing. The detection limit of such electrodes is ~1 nM (Lanter et al., 1982). The macro PVC electrodes were used to check the final concentrations of free Ca in the calibrating solutions. The PVC membranes were a gift of Dr. D. Ammann (ETH, Zurich, Switzerland).

CALIBRATION SOLUTIONS Table I shows the composition of the calibration solutions used to test the Ca microelectrodes. Solution pCa 2 is the ASW used to superfuse the nerve; SO_4^{2-} (as $MgSO_4$) was omitted because it precipitates Ca^{2+}. There is no noticeable change in the physiology of the photoreceptors bathed in SO_4^{2-}-free ASW, as shown by Martinez and Srebro (1976). In pCa 3, the decrease in free Ca was compensated by an increase in Na^+ to keep the ionic strength constant. Above pCa 3, Ca buffers were used and the concentrations of Na^+ and K^+ were varied in order to simulate the intracellular environment; tetramethylammonium chloride (TMA; 87720 Fluka) was added to keep the ionic strength constant. $[Na^+]$ and $[K^+]$ above pCa 3 were set at values close to those measured intracellularly in *Balanus* photoreceptors using Na^+- and K^+-selective microelectrodes (H. M. Brown, 1976). Mg^{2+} was not added, because it complicates the calculation of $[Ca^{2+}]$ in buffered solutions and because it has a negligible interference on E_{Ca} (see Fig. 2*B*).

The apparent association constants of the buffers used were determined empirically by a method similar to the one used by Allen et al. (1977) with aequorin. The principle consists of calibrating a Ca macroelectrode in two different concentrations of nonbuffered Ca solutions, which is sufficient to set the slope of the calibration curve, since the Ca macroelectrode has a Nernstian slope down to pCa 7. Then, for each ligand, the free Ca of a buffered Ca solution with a ratio of bound Ca per free ligand of 1 is measured and aligned on the slope set previously (Levy, 1979). The pH is then modified (see Portzehl et al., 1964) to values reasonably close to intracellular pH (Levy and Coles, 1977) in order to have whole-number pCa values with ratios of bound Ca per free ligand of 1. The logs

of the K_a of NTA, H·EDTA, EGTA, and EDTA at an ionic strength of ~0.6 M were found to be, respectively, 4.0 (pH 7.60), 5.0 (pH 6.90), 6.0 (pH 6.86), and 7.0 (pH 7.0). Modifying the pH, instead of the ratio of bound Ca per free ligand, was chosen to provide the maximum buffering capacity.

CALIBRATION OF THE Ca MICROELECTRODES The microelectrodes were first conditioned by immersing their tips for 0.5–3 h in the pCa 6 solution, as recommended by Dr. D. Ammann (personal communication). They were then calibrated in a continuous flow of solutions in a Plexiglas chamber. Fig. 2*A* shows a recording of the actual calibration

TABLE I

Composition of the Calibration Solutions

pCa	2 (ASW)	3	4	5	6	7	∞
NaCl	435	462	20	20	20	20	20
K+*	10	10	120	120	120	120	120
$MgCl_2$	53	53	—	—	—	—	—
$CaCl_2$	10	1	5	5	5	5	—
NTA	—	—	10	—	—	—	—
H·EDTA	—	—	—	10	—	—	—
EGTA	—	—	—	—	10	—	—
EDTA	—	—	—	—	—	10	10
TMA	—	—	465	465	465	465	465
Tris	10	10	—	—	—	—	—
K·HEPES	—	—	10	—	—	—	—
K·PIPES	—	—	—	10	10	—	—
K·BES	—	—	—	—	—	10	10
pH	7.80	7.80	7.60	6.90	6.86	7.00	7.00

NTA: nitrilotriacetic acid.

H·EDTA: *N*-hydroxyethylethylenediaminetriacetic acid.

EGTA: ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

Tris: tris(hydroxymethyl) aminomethane.

HEPES: *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

PIPES: piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

BES: *N*,*N*-bis[2-hydroxyethyl]-2 aminoethane sulfonic acid.

TMA: tetramethylammonium chloride.

* Total [K^+] includes KCl plus KOH, used to bring the solution to the desired pH, and K^+ contained in the pH buffer stock solution (e.g., K·BES).

of a Ca microelectrode and Fig. 2*C* shows the calibration curves of four different Ca microelectrodes actually used for intracellular recordings. One can see that the slope is Nernstian up to pCa 6; then the emf change is ~20–22 mV between pCa 6 and 7 and ~15–26 mV between pCa 7 and pCa infinity.

Although the Ca microelectrodes measure Ca activities, the measured Ca_i is expressed as the intracellular free Ca concentration. On the assumption that the extracellular medium has the same ionic strength as the intracellular one, and since care was taken to calibrate the Ca electrodes in solutions that had a constant ionic strength similar to the intracellular fluid, the Debye-Huckel activity coefficient of Ca^{2+} should remain constant.

RESPONSE TIME OF THE Ca MICROELECTRODES The inset of Fig. 2*C* shows sample recordings of the response times of a Ca microelectrode. The three free Ca concentrations used to measure the response time were chosen to simulate the Ca_i changes typically observed during this study. The top trace shows that the electrode reached 50% of its final potential in 75 ms (t_{50}) for a free Ca concentration change from 1 (pCa 6) to 10 μM

(pCa 5); the t_{90} was 150 ms. The bottom trace shows that the t_{50} and t_{90} are, respectively, 1 and 3 s for a change from 0.1 to 1 μM. The t_{50} for a change between 1 and 10 μM varied between 65 and 500 ms for all Ca microelectrodes used.

INTERFERENCE FROM OTHER IONS The selectivity of the ligand ETH 1001 for Na^+, K^+, H^+, and Mg^{2+} was tested under the present conditions. Fig. 2*B* shows results of

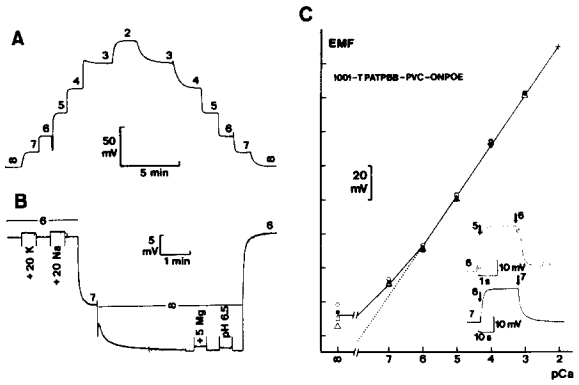

FIGURE 2. (*A*) Actual calibration of a Ca microelectrode that was used for intracellular recordings in two *Limulus* ventral photoreceptors. The numbers on each baseline correspond to the pCa of the calibrating solutions (Table I). The time taken for the emf to equilibrate after each solution change is faster for increasing than for decreasing concentrations because of washout and diffusion. As can be seen, hysteresis is minimal. (*B*) Interference of Na^+, K^+, Mg^{2+}, and H^+ on the measurement of Ca^{2+} with a Ca microelectrode. Interference was tested at low free Ca concentrations to simulate experimental conditions. Numbers above the recording correspond to the pCa of the calibrating solutions. The pCa infinity solution used had 10 mM EGTA (no Ca ions added). The 5 mM Mg^{2+} solution had 6.5 mM total Mg^{2+}. The chart recorder was stopped between each solution change. (*C*) Calibration curves of four different Ca microelectrodes actually used for intracellular recordings. The ordinate shows the potential measured by the Ca electrode; the abscissa shows the concentration of Ca^{2+} in the different calibrating solutions, where pCa = $-\log Ca^{2+}$ concentration. The dashed line shows the Nernst slope (29.5 mV per pCa unit). The emf at pCa 2 was set to the same value for ease of comparison. The formula shown on the top left refers to the composition of the PVC-gelled sensor used. The inset shows the response time of a Ca microelectrode suitable for intracellular recording. The top trace shows the response time of the Ca electrode when the Ca solution was switched from 1 (pCa 6) to 10 μM (pCa 5) and back to 1 μM. The lower trace shows the response of the same Ca electrode when the Ca solution was changed from 0.1 to 1 μM and back to 0.1 μM (pCa 7). The solutions were rapidly changed using pneumatic valves in a manner described by Coles and Tsacopoulos (1979). The resistance of the Ca microelectrode was 4.7×10^{11} Ω.

the interference of the ions Na^+, K^+, H^+, and Mg^{2+} upon measurement of free Ca in vitro. Increasing Na^+ by 20 mM to a pCa 6 solution resulted in an increase in emf of ~1.5 mV, whereas adding 20 mM K^+ resulted in an increase of ~1 mV. Decreasing the pH by 0.5 log units in pCa infinity resulted in <1 mV change in the emf, whereas increasing $[Mg^{2+}]$ by 5 mM resulted in an increase of ~1 mV. (Varying the pH and $[Mg^{2+}]$ in pCa 6 solution would have resulted in an actual change of $[Ca^{2+}]$.)

RESULTS

Measurement of Ca_i

The protocol for measuring Ca_i in *Limulus* ventral photoreceptors was as follows. After the Ca microelectrode had been properly calibrated (see Methods and Fig. 2), it was put in the bath, along with the V microelectrode, and allowed to equilibrate. Photoreceptors that appeared to be single, isolated cells were first impaled with the voltage electrode. The sensitivity profile of the cell was then determined by means of a microspot (see Methods). The cell was then impaled with the Ca electrode either in the most sensitive region or away from it (see *Spatial Localization of the Light-induced Ca_i Increase*). After cell penetration, the Ca electrode potential was allowed to equilibrate in the dark while the sensitivity of the cell was monitored using 20-ms-long test flashes of uniform illumination at a frequency of 0.1 Hz. It usually took 10–30 min for Ca_i to reach a stable value. Fig. 3 shows a typical experiment that followed this protocol. The lower trace (E_{Ca}) in Fig. 3 shows the potential measured by the Ca electrode: inside the cell, E_{Ca} is the sum of the emf generated by the free Ca concentration and the photoreceptor transmembrane potential (see Methods). The middle trace (E_m) shows the transmembrane potential measured by the voltage electrode. The top trace ($E_{Ca} - E_m$) shows the Ca signal, which is converted to free Ca concentration using the calibration curve for that particular Ca electrode (see Fig. 2*C* for a typical calibration curve). During the interval labeled *a* in Fig. 3 (40 min), the cell was impaled with the voltage electrode and its sensitivity profile was determined; the Ca microelectrode was then placed against the cell membrane in the region of highest sensitivity. At the time marked by the uppermost arrow, a single brief voltage pulse was applied to the jolter, which resulted in the impalement of the cell by the Ca microelectrode. This caused the cell to depolarize by ~28 mV and to transiently lose sensitivity by >0.6 log units. To ensure that both electrodes were in the same cell and also to measure the membrane input resistance, current was passed through the voltage electrode; the legend of Fig. 3 explains the procedure for measuring the input resistance of the cell. After the interval labeled *b* in Fig. 3 (10 min), when Ca_i had equilibrated, a bright 20-ms-long flash (lowermost arrow) elicited a large transient increase in Ca signal. During the recovery of Ca_i to the dark level after the flash, the sensitivity of the cell was monitored by varying the intensity of the test flashes.

The sensitivity of the cell was monitored during the whole time that the experiment lasted. For the cells included in this study, the dark-adapted sensitivity, S_D, was variable with time; S_D would sometimes be reduced by 0.1–1.0 log unit or on the other hand be increased by 0.1–0.5 log unit over the 2–6 h that the experiment lasted. The decrease in S_D often occurred after a bright adapting light was given. At the end of an experiment, both electrodes were withdrawn; E_{Ca} was then checked for drift, and E_m for a change in tip potential. Because the Ca signal is a differential measurement, any drift in the voltage electrode potential (typically 2–3 mV) was taken into account in deciding whether the Ca electrode had drifted. The drift of E_{Ca} was typically 2–10 mV, over a period of 2–6 h. The Ca electrode was then recalibrated in the calibration chamber. Almost always, the calibration curve measured after was identical to that measured

before between pCa 2 and pCa 6.0; however, there was a variable loss of sensitivity above pCa 6.0 of ~5–10 mV per pCa unit. Since the measured level of Ca_i was almost always above 1 μM, this increase in the detection limit did not affect the measured values of Ca_i.

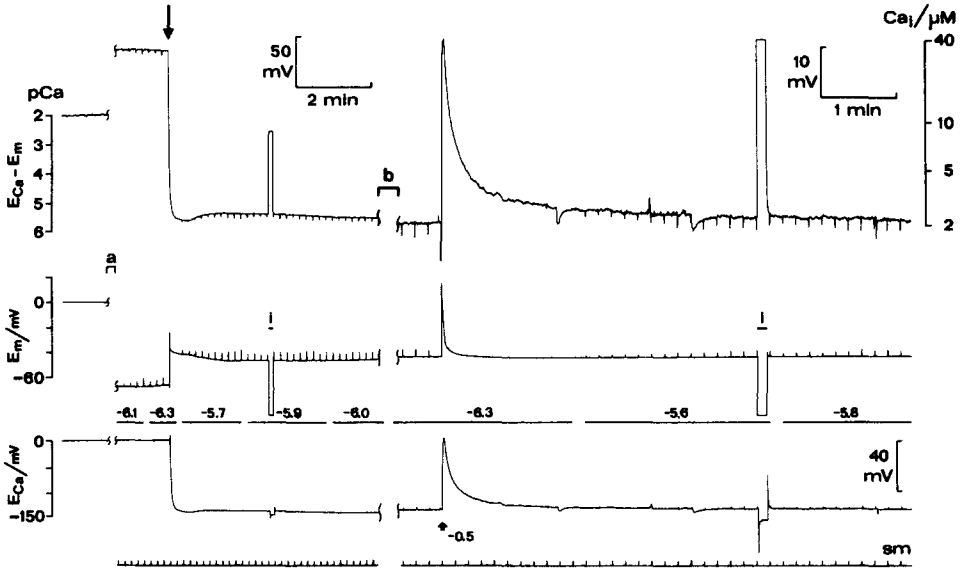

FIGURE 3. Impalement of a *Limulus* ventral photoreceptor with a voltage microelectrode and a Ca microelectrode. Top trace (Ca signal): potential difference between the Ca electrode and the voltage electrode. Middle trace (E_m): membrane potential measured by the voltage electrode. Lower trace (E_{Ca}): potential measured by the Ca electrode. The top left scale corresponds to the calibration curve of that electrode; the top right scale is an expanded portion of it. During the interval marked by *i*, a −1-nA current was passed through the voltage electrode; this resulted in a voltage drop across the V electrode that is made up of both the voltage drop across the input resistance of the cell and that of the electrode, whereas the steady voltage drop measured by the Ca electrode is solely a measure of the input resistance of the cell (~9 MΩ here); the fast transients on the E_{Ca} voltage drop are capacitative artifacts. The bars under the E_m record indicate the time during which 20-ms test flashes of the indicated log intensities were given. The test flash was repeated every 10 s and was used to measure the sensitivity of the cell. On the Ca signal trace, the fast negative-going potentials resulting from the test flashes or the adapting flash are electrical artifacts caused by the difference in electrical time constants of the two electrodes. Note the change in time scale and amplitude scale after interval *b*. sm, stimulus monitor.

The sensitivity of the cell, S_D, was used as a criterion in selecting photoreceptors for study. The 31 cells described here had log S_D values between 4.6 and 7.2 (see Fig. 4*A*). Photoreceptors impaled with only a fine voltage microelectrode have a somewhat higher log S_D, between 8.2 and 6.2. The fact that the cell in Fig. 3 depolarized without a great loss in sensitivity is taken as an indication that the cells were probably not unduly damaged upon impalement by the Ca

electrode. In some cells, after Ca_i and E_m had equilibrated for 1 or 2 h, spontaneous reductions or increases of the resting transmembrane potential were seen without any noticeable change in Ca_i. Another indication that most of the cells were not unduly damaged was the presence of quantum bumps in the dark.

Resting Ca_i

Photoreceptors with log S_D values of $\geq$4.6 had a mean Ca_i of 3.5 μM $\pm$ 2.7 SD (n = 31). This value seems to be higher than most published values of resting Ca_i in neurons (H. M. Brown and Owen, 1979; Alvarez-Leefmans et al., 1981; Gorman et al., 1984). In most neurons, low membrane potentials are often taken as an indicator of cell damage. Alvarez-Leefmans et al. (1981) found that *Helix* neurons with a reduced resting membrane potential had a somewhat higher Ca_i. As mentioned in the preceding section, variations in the value of the resting membrane potential in *Limulus* ventral photoreceptors (in the range −45 to −70 mV) do not seem to be an indicator of cell damage. Indeed, in the 31 cells reported here, there was no apparent relationship between resting Ca_i and resting membrane potential (not shown). Cells with a higher resting membrane potential did not necessarily have the lowest Ca_i, which agrees with similar measurements made in drone photoreceptors (Levy, 1979).

A correlation was therefore sought between Ca_i and S_D, which is a more useful indicator of photoreceptor function. Fig. 4*A* shows a scatter diagram of Ca_i vs. $\log(1/S_D)$. In this plot, the most negative values on the abscissa represent the most sensitive cells. One can see that there is no marked relation between Ca_i and $\log(1/S_D)$. In Fig. 4*A*, the different symbols correspond to the different locations of the Ca microelectrode impalement within the photoreceptors (see *Spatial Localization of the Light-induced Ca_i Increase*); resting Ca_i seems to be homogeneous throughout the cell. If resting membrane potentials and S_D are not good indicators of resting Ca_i, perhaps the input resistance of the cell at rest is. The value of the input resistance, R_i, should give an indication of how leaky the photoreceptors are after their impalement with two microelectrodes. Fig. 4*B* shows a scatter diagram of Ca_i vs. R_i; here lower values of Ca_i seem to be associated with higher values of R_i. The approximately 10-fold variation in resting Ca_i among the 31 cells studied here appears to be otherwise unrelated to resting membrane potential, S_D, or R_i.

Spatial Localization of the Light-induced Ca_i Increase

During our first experiments, apparent large variations from cell to cell were seen in the magnitude and time course of the light-induced Ca_i increases. Occasionally, a large and fast increase in the Ca signal would be recorded, but most often the increase was small and slow, and, in half of the cells (n = 10), the Ca microelectrode did not detect any light-induced Ca_i increase. Since the Ca microelectrode measures a very localized volume of the cell, one possible explanation for these variations could be that the Ca electrode was not always near the site where the increase in Ca_i occurred (Levy and Fein, 1983). Recently, it was shown that ventral photoreceptors have two distinct functional lobes (Stern et al., 1982; Calman and Chamberlain, 1982). Stern et al. (1982) have shown that the sensitivity to light is highest in the so-called rhabdomeral lobe (R lobe),

while the arhabdomeral lobe (A lobe) is relatively insensitive. We provide evidence below that the light-induced Ca_i increase is greatest in the region of the cell that has the highest sensitivity.

The A and R lobes can easily be seen if one strips the cells of their glia. We found it easier to scan the cell with a microspot while recording intracellularly (see Methods). Once the sensitivity profile was determined, the cell was impaled with the Ca microelectrode either in the most sensitive area or away from it.

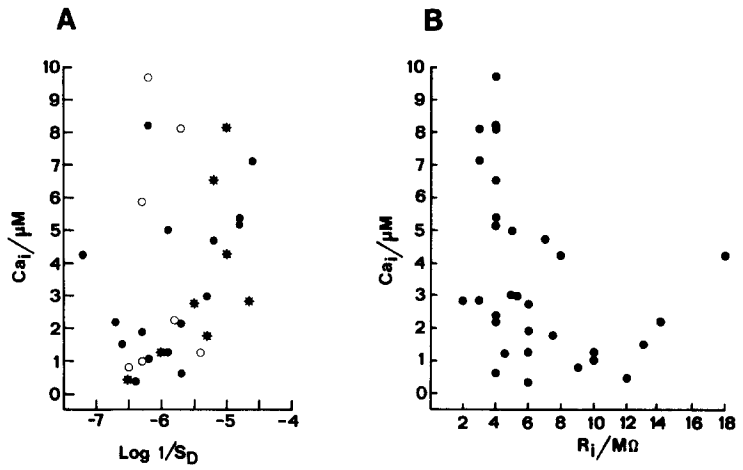

FIGURE 4. (*A*) Scatter diagram of measured resting Ca_i plotted against the log 1 per sensitivity of the photoreceptor in the dark-adapted state ($1/S_D$); the most negative values represent the most sensitive cells. Sensitivity is defined in Methods. Ca_i and sensitivity were measured after both values had equilibrated in the dark after impalement. The different symbols refer to the different regions of the cell impaled by the Ca microelectrode. Each symbol represents one photoreceptor. Filled symbols: the most sensitive region. Open symbols: 30–60 μm away from the most sensitive region. Stars: unknown location from experiments where the sensitivity profile of the cell was not determined. (*B*) Scatter diagram of measured resting Ca_i plotted against the input resistance of the photoreceptor at rest (R_i). The input resistance was measured by passing a hyperpolarizing current of 1 nA through the voltage electrode and recording the steady voltage change measured by the Ca electrode (see Fig. 3).

The terms "most sensitive area" and "away from it" are used rather than "R lobe" and "A lobe" because the lobes were not usually distinguishable. However, by carefully orienting the photoreceptors on the side of the nerve, it was sometimes possible to see two lobes through the light microscope (see Fig. 1).

Fig. 5*A* shows a light-induced Ca_i increase measured by a Ca microelectrode located ~60 μm away from the most sensitive area. A bright step of log relative intensity −1.0 (uniform illumination) elicited a Ca_i increase of <1 μM, with a time to peak of ~10 s. The sensitivity profile of the cell is shown on the right, as determined at the end of the experiment before the Ca and the V microelectrodes were withdrawn. The distance between the Ca electrode and the most sensitive

site was measured by using the calibrated micropositioner that moves the microspot (see Methods).

Fig. 5*B* shows a light-induced Ca_i increase measured by a Ca electrode inserted at the most sensitive area. A 20-ms flash of log relative intensity −1.0 (uniform illumination) elicited a large increase in the Ca signal with a time to peak of ~1 s, which corresponds to a Ca_i increase of ~30 μM. The inset shows a drawing of the cell and the position of the Ca electrode as seen through the light microscope; responses to microspot flashes of constant intensity are represented as vertical lines. Since the sensitivity started decreasing toward the middle of the cell, and since the intent was to record Ca_i at the most sensitive area, the scanning was interrupted and the cell was impaled at that site.

The location of the most sensitive area was not always at one end of the cell. Sometimes a cell would have an almost uniform sensitivity from one end to the other, a situation that might occur if the R lobe occupied a position all along the length of the cell (Calman and Chamberlain, 1982). The light-induced Ca_i increases in such cells were either very large or very small (see Levy, 1983).

Table II shows that the peak amplitude and the time to peak of the light-induced Ca_i increase (ΔCa_i) depend strongly on the location of the Ca electrode in the cell. Small and slow Ca_i increases are observed in regions that have relatively low sensitivity compared with other regions of the same cell. Large and fast ΔCa_i values are associated with regions of relatively high sensitivity. When the intent was to record Ca_i away from the most sensitive areas, the measured ΔCa_i value was always much smaller and slower than the values recorded in the highest-sensitivity sites (cells 1, 3, 12, and 16). The reverse was not always true, since cells with uniform sensitivity profiles sometimes yielded slow and small Ca_i increases (cells 6, 7, and 13). In the Discussion, we provide a possible explanation for this apparent inconsistency in the results. In the case of cell 4 (Table II), which had an R lobe localized at one end of the cell (as in Figs. 1 and 5*B*), the cell was impaled by the Ca microelectrode ~30 μm from the site of highest sensitivity, but at a site where sensitivity was only 0.3 log units lower. Even so, the light-induced Ca_i increase was comparatively large and fast. This is not surprising since the R lobe has been shown to extend over tens of microns (Stern et al., 1982; Calman et al., 1982; Payne and Fein, 1983; see also Fig. 1). As also shown in Table II, the average light-induced Ca_i increase was ~20 times larger and faster in the most sensitive area than it was away from it.

Unless otherwise indicated, all the data presented below were obtained from cells that were impaled with the Ca electrode in the most sensitive region.

Effect of Stimulus Energy on the Light-induced Ca_i Increase

Fig. 6*A* shows a series of Ca_i increases induced in a dark-adapted photoreceptor by brief stimuli of increasing intensities. The data show that the change in Ca_i (ΔCa_i) is graded with light intensity over 3 log units of stimulus energy. Assuming that one discrete wave results from the photoisomerization of one rhodopsin molecule (Rh*), and using the physiological calibration of the light stimulus (see Methods), we calculate that ~100 Rh* are needed to produce a detectable ΔCa_i for log relative intensity −4.5. Similarly, ΔCa_i saturates at a log relative intensity of −1.5, which corresponds to ~10^5 Rh*.

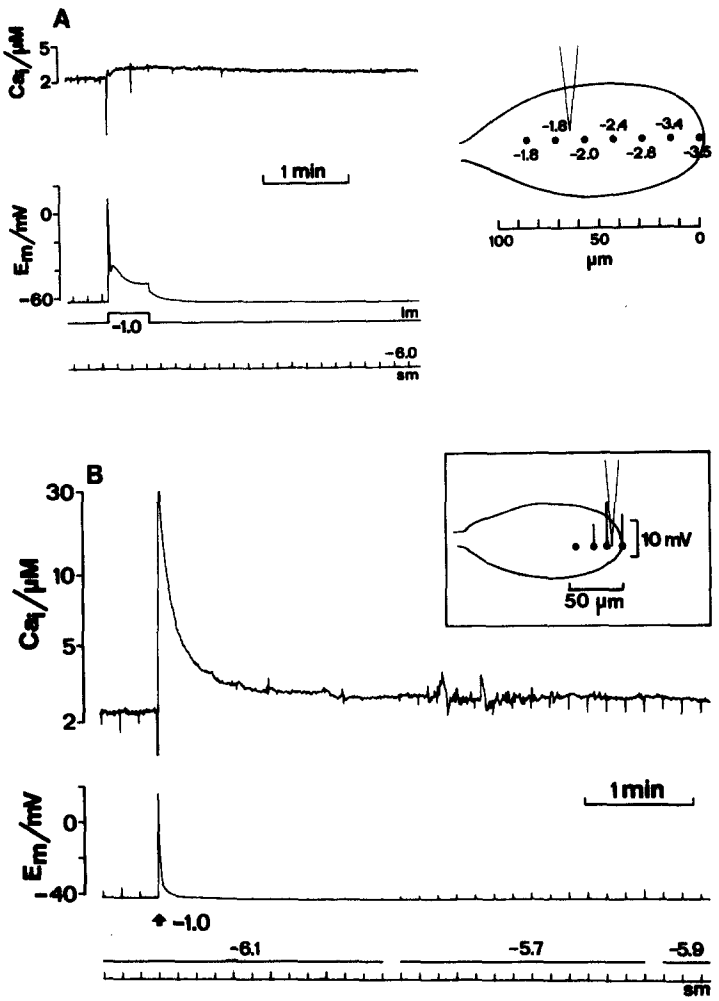

FIGURE 5. Nonuniformity of the light-induced Ca_i increase. The top trace is the Ca signal; the E_m trace is the membrane potential measured by the voltage electrode. 20-ms test flashes (uniform illumination) whose log intensity values are shown above the stimulus monitor (sm) trace were presented throughout. (*A*) Light-induced Ca_i increase measured away from the most sensitive area of the cell. The photoreceptor was first scanned with the microspot; then the cell was impaled with the Ca electrode ~60 μm away from the most sensitive area. At the end of the experiment, with both electrodes still in the cell, the scanning was repeated and the distance between the Ca microelectrode and the most sensitive area was redetermined to give the distances shown in the schematic drawing at the right. Each dot with its corresponding log $(1/S_D)$ gives the location of the microspot during the actual scanning. The voltage electrode is not shown. lm, light monitor (uniform). Cell 16 of Table II. (*B*) Light-induced Ca_i increase measured in the region of highest sensitivity. The top trace shows the Ca signal; the E_m trace shows the membrane potential measured by the voltage electrode. The arrow shows the time when a 20-ms bright flash (uniform illumination) of log relative intensity −1.0 was presented. The inset shows a schematic drawing of the photoreceptor and the Ca microelectrode as observed through the light microscope. Each dot represents the location of the microspot during the

TABLE II

Spatial Localization of the Light-induced Ca_i Increase

Cell	(A) Ca microelectrode distant from the site of Ca_i increase: log(1/S_D) Uniform illumination	log(1/S_D) Microspot: High-sensitivity site	log(1/S_D) Microspot: Ca electrode site	Photostimulation (uniform): Distance*	Photostimulation (uniform): Log relative intensity	Duration	Peak‡ ΔCa_i	t_p§
				μm		*s*	*μM*	*s*
1‖	−6.2	−4.5	−2.7	74	−0.5	83	2.40	17
3	−6.2	−3.6	−2.1	66	−0.5	0.02	2.40	14
6¶	−6.3	−4.6	**	**	−0.5	0.02	0.78	16
7¶	−6.6	−2.0##	**	**	−0.5	0.02	0.78	16
12	−6.3	−4.5	−3.4	57	−0.5	62	0.0	—
13	−5.5	−2.5	**	**	−1.5	0.02	2.87	33
16	−6.0	−3.5	−1.9	63	−1.0	30	0.60	10
						Average	1.32	15.3
						SD§§	0.94	1.2
	(B) Ca microelectrode close to the site of Ca_i increase							
2‖	NM	−3.9	−3.9	0	0.0	180	33.5	2.0
4	−4.6	−2.4	−2.1***	28	−0.5	0.02	11.9	1.2
5	−5.6	−4.5	−4.5	0	−0.5	0.02	39.5	2.0
8¶	−5.7	−4.6	−4.6	0	−0.5	0.02	21.3	0.5
9	−6.3	−2.6‖‖	−2.6‖‖	0	−0.5	0.02	35.9	1.5
10	−6.6	¶¶	Same	0	−1.0	0.02	26.7	1.0
11	−6.3	−4.5	−4.5	0	−1.5	0.02	40.6	1.0
14	−7.2	¶¶	Same	0	−1.5	0.02	44.1	0.3
15	−6.2	¶¶	Same	0	−0.5	0.02	22.0	4.0
						Average	26.1	1.8
						SD§§	11.4	1.3

* Distance between Ca electrode and highest-sensitivity site.

‡§ Respectively, peak amplitude and time to peak of the light-induced Ca_i increase.

‖ Same Ca microelectrode used in two neighboring cells.

¶ Same Ca microelectrode used in three neighboring cells.

** Unknown, because the cell had a uniform sensitivity.

Criterion was an incremental response of 4–5 mV resulting from a test flash of log intensity −2.0 superposed on a uniform background light of log relative intensity −4.5.

§§ Average of cells where the log intensity and the duration of the stimulus were, respectively, −0.5 and 0.02 s.

‖‖ Same as above, but the log relative intensity of the test flash and of the uniform background light were, respectively, −2.6 and −5.0.

¶¶ The cell was scanned rapidly for the highest-sensitivity site and impaled at that site.

*** This cell did not have a uniform sensitivity profile.

NM, not measured.

scanning. The vertical lines arising from the dots correspond to incremental responses to 20-ms test flashes (microspot) of log relative intensity −2.6 superposed on a steady and uniform background light of log relative intensity −5.0. The steady background light was used because the threshold response amplitude was too variable in the dark-adapted state. The voltage electrode is not shown. Cell 9 of Table II.

It should be kept in mind that the Ca electrodes used in this study typically responded too slowly (see Methods) to faithfully reproduce the initial phase of the Ca increase. Thus, the rise time of the Ca_i increase is overestimated, while the peak amplitude is underestimated (Fig. 6*B*).

Relationship Between the Light-induced Ca_i Increase and Sensitivity

In this section, we are concerned with determining whether a direct relationship exists between the desensitization and the Ca_i increase that occurs during light adaptation. Light adaptation can easily be quantified over a broad range of light intensities using the increment-threshold curve (see, e.g., Lisman and Strong, 1979). Therefore, simultaneous measurements of incremental light responses

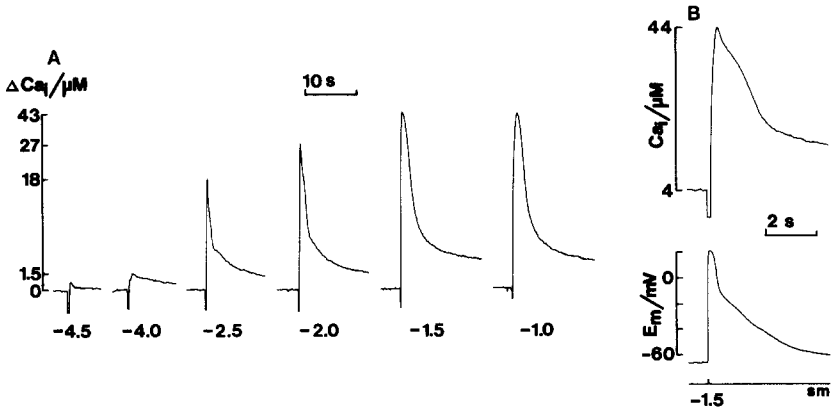

FIGURE 6. (*A*) Effect of stimulus energy on the light-induced Ca_i increase. Only the Ca signals are shown. The left scale gives the amplitude of the peak ΔCa_i. The log relative intensity of each 20-ms stimulus is given under each trace. The beginning of the negative-going electrical artifact gives an indication of the time of occurrence of the stimulus. The cell was allowed to dark-adapt before each flash. The dark-adapted log threshold was −7.2. (*B*) Simultaneous recording of Ca signal (top trace) and membrane potential (middle trace) on a faster time scale than in *A*. sm, stimulus monitor; same flash duration and same cell as in *A*. This record was taken before the series of flashes shown in *A*.

and Ca signals were made for increasing intensities of steady background illumination. The range of background intensities used extended over 4 log units, starting with intensities that could barely desensitize the cell. These experiments were analyzed for the following information: (*a*) the effect of increasing background light intensity on Ca_i; (*b*) the Ca_i increase at the threshold of adaptation; and (*c*) the relationship between the light-induced rises in Ca_i and threshold.

Fig. 7 shows an experiment from which the above information was extracted. Each background light can be seen to induce a decrease in sensitivity and an increase in Ca signal. At each light intensity, the goal was to make the duration of the background light long enough to allow the Ca signal and sensitivity to reach stable values. At the end of the last background light (log $I_b = -3.0$), the

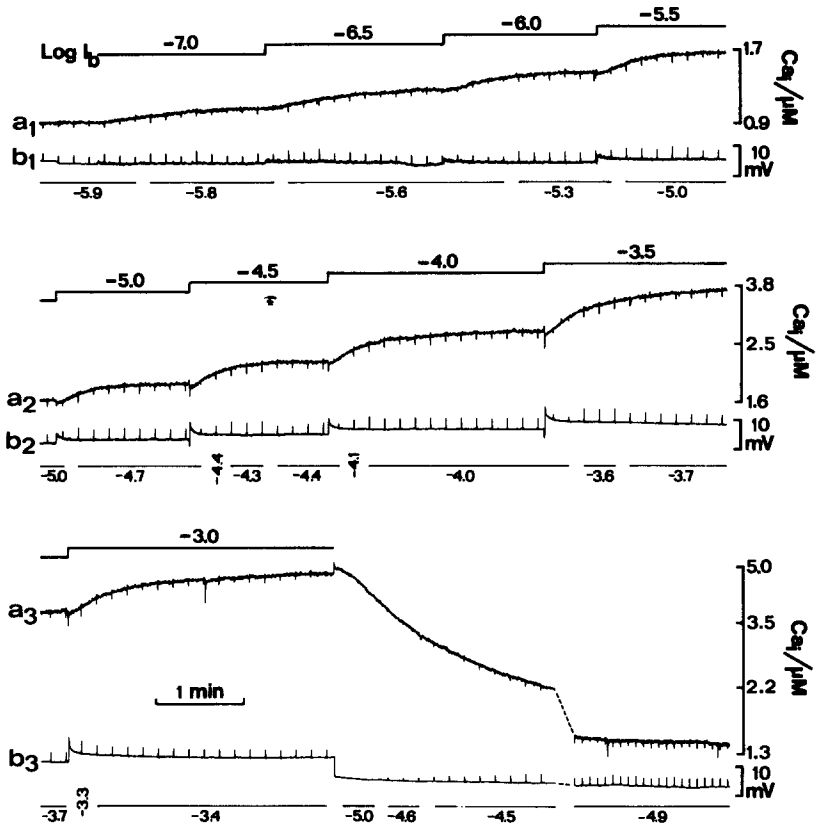

FIGURE 7. Relationship between light-induced rises in Ca_i and threshold. Typical experiment showing the effect of increasing background intensities on Ca_i and threshold. Traces a_1, a_2, and a_3 are the Ca signal; the scales on the right of the traces correspond to the calibration curve of the Ca microelectrode used. Traces b_1, b_2, and b_3 are the membrane potential recorded by the V electrode. The traces are shown below each other solely for convenience. The trace above the Ca signals, together with its associated numbers, corresponds to the occurrence and intensities of the background light. The bars below the voltage record, together with their associated numbers, correspond to the log relative intensities of the 20-ms test flashes, which elicited a criterion response of ~4 mV. The positive-going potential on the Ca signal record at the end of the brightest light is an electrical artifact resulting from the fact that the Ca electrode and the voltage electrode were not quite isopotential. The dashed line during dark adaptation represents a deleted portion of the record of 80 s; note that the chart recorder speed was halved after the deletion. To ensure the reproducibility of these results, the following measurements were made. Before this record was taken, the cell was stimulated twice with a steady background light of log intensity −6.5; both light stimulations elicited a reversible steady increase in Ca_i similar to that shown. After this record was taken, stimulation with a steady background light of log intensity −4.0 elicited a similar steady increase in Ca_i and a similar decrease in sensitivity as measured during this record. Cell 1 of Table III.

cell was allowed to dark-adapt. This paradigm was chosen to minimize the duration of the experiment within the expected life of the preparation.

EFFECT OF BACKGROUND INTENSITY ON Ca_i Fig. 8*A* shows a plot of the relative increase in Ca_i vs. the background intensity. Data points correspond to

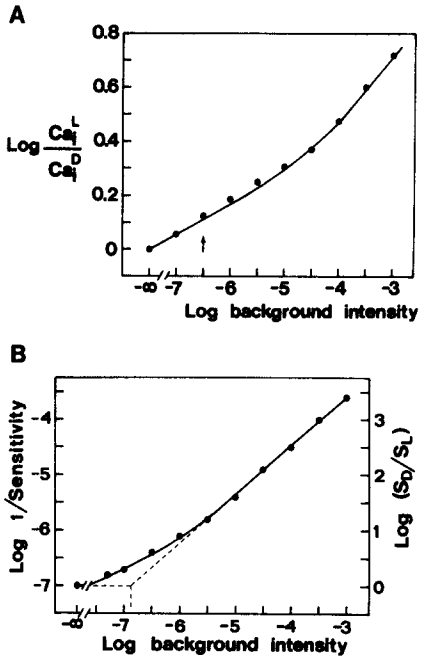

FIGURE 8. (*A*) Plot of the relative increase in Ca_i vs. background intensity. Ca_i^L is the absolute Ca_i measured after it had reached a stable value during illumination. Ca_i^D is the dark-adapted value of Ca_i obtained before the ascending series of background lights used in Fig. 7. Data points correspond to measured values of Ca_i at each background intensity taken from the cell of Fig. 7. The arrow indicates the light intensity of the threshold of adaptation (determined as shown in *B*), which elicited a steady Ca_i increase in this cell (cell 1, Table III). Ca_i^D was 0.93 μM. (*B*) Sample increment threshold curve obtained using the experimental procedure of Fig. 7. The left-hand ordinate shows the log threshold; the right-hand ordinate shows the corresponding relative desensitization. S_D and S_L refer to the values of sensitivity measured in the dark- and light-adapted states, respectively. At higher intensities, the linear portion has a slope n of 0.90 (cell 5 of Table IV). The dashed lines show the intersection of the two portions of the incremental threshold curve used to determine the threshold of adaptation (see text).

measured values of Ca_i at each background intensity, taken from the cell of Fig. 7. Ca_i^L refers to the absolute Ca_i measured after it appeared to reach a stable value during the steady illumination. Ca_i^D refers to the absolute value of Ca_i in the dark-adapted state measured before the ascending series of adapting lights.

Table III tabulates for five cells the changes in Ca_i induced by steady background light, using the same experimental procedure as in Fig. 7. The relative

TABLE III

Effect of Different Background Intensities on the Relative Increase in Ca_i and on Desensitization (S_D/S_L)

			Changes induced by log I_b					
			−6.5		−5.0		−3.5	
Cell	Ca_i^P	$\log(1/S_D)$	Ca_i^L/Ca_i^P	$\log(S_D/S_L)$	Ca_i^L/Ca_i^P	$\log(S_D/S_L)$	Ca_i^L/Ca_i^P	$\log(S_D/S_L)$
	μM							
1	0.93	−5.9	1.33	0.3	2.02	1.1	4.00	2.2
2	1.89	−6.3	1.22	0.3	1.76	1.3	4.05	2.5
3	2.98	−5.3	1	0.1	1.20	0.8	1.98	1.8
4	0.59	−5.2	1	0	1.36	0.5	2.66	1.7
5	4.55	−7.0	1.08	0.6	1.18	1.6	1.62	3.0

increase in Ca_i was somewhat variable from cell to cell, but in all the cells Ca_i increased monotonically with the log intensity of the background light.

RELATIONSHIP BETWEEN LIGHT-INDUCED Ca_i INCREASE AND DESENSITIZATION Fig. 8*B* shows a representative incremental threshold curve obtained simultaneously with the light-induced incremental Ca_i rise. The ordinate shows the log threshold, both on an absolute and on a relative scale. Dim background intensities slightly desensitize the cell; with higher intensities, the log desensitization is linearly related to the log background intensity. The slope (n) of the linear portion for five cells is given in Table IV; it had an average of 0.76 ± 0.10 (SD), which is similar to the slopes measured by Lisman and Strong (1979) (see also Wong, 1978). The intersection between the linear portion of the incremental threshold curve and the horizontal portion where desensitization is zero can be defined as the threshold of adaptation (dashed lines on Fig. 8*B*).

Fig. 9 shows the relationship between the relative increase in Ca_i and desensitization on a log-log plot for the five cells in Table IV. The data points for each cell were fitted by eye with a straight line, which implied that:

TABLE IV

Relationship Between the Light-induced Ca_i Increase and Desensitization

			Threshold of adaptation		Ca_i^L/Ca_i^P for a 100-fold change in sensitivity
Cell	n	m	log I_b	Ca_i^L/Ca_i^P	
1	0.70	0.26	−6.5	1.33	3.3
2	0.77	0.24	−6.7	1.22	3.0
3	0.65	0.16	−5.9	1.09	2.1
4	0.80	0.25	−5.6	1.20	3.2
5	0.90	0.07	−6.9	1.05	1.4
Mean	0.76	0.20		1.18	2.6
SD	0.10	0.08		0.11	0.8

n = slope of the increment threshold curve (see Fig. 8*B*).

m = slope of $\log(Ca_i^L/Ca_i^P)$ vs. $\log(S_D/S_L)$ (see Fig. 9).

I_b = background illumination at the threshold of adaptation determined by intersection on incremental threshold curve as shown in Fig. 8*B*.

$$\log(Ca_i^L/Ca_i^D) = m \log(S_D/S_L); \quad (1)$$

or that

$$(Ca_i^L/Ca_i^D)^{1/m} = S_D/S_L. \quad (2)$$

Table IV gives the values of m for each cell: the average m for the five cells was 0.20 (range 0.26–0.07). The relationship of Eq. 2 indicates that the higher the Ca_i, the more desensitized the cell becomes. The range of values observed for m indicate a remarkably steep relationship between the measured increase in Ca_i and the desensitization. The last column of Table IV shows that a 100-fold decrease in sensitivity is associated with an increase in Ca_i between 1.4- and 3.3-fold. In other words, a light-induced desensitization of 2 log units is associated with an increase in Ca_i from, say, 1 μM in the dark-adapted state to ~3.5 μM during illumination.

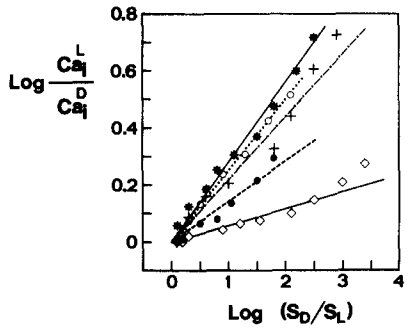

FIGURE 9. Relationship between sensitivity and Ca_i. Log-log plots of the relative increase in Ca_i vs. desensitization for five photoreceptors. The curves have the form $(Ca_i^L/Ca_i^D)^{1/m} = S_D/S_L$. The data points for each cell were fitted by a straight line of slope m. Table IV gives the value of m for each photoreceptor. Stars: cell 1; plusses: cell 2; solid circles: cell 3; open circles: cell 4; diamonds: cell 5.

DETERMINATION OF THE RELATIVE RISE IN Ca_i AT THE THRESHOLD OF ADAPTATION Table IV gives the value of the background intensity at which adaptation starts to occur and the corresponding relative increase in Ca_i. The log background intensity at the threshold of adaptation was determined in each cell, as illustrated in Fig. 8*B*. The corresponding relative increase in Ca_i was then determined for each cell using the relationship in Fig. 8*A*. On the average, Ca_i increased by ~20% from its resting value.

Effect of Light Adaptation on the Light-induced Ca_i Increase

J. E. Brown and Blinks (1974), using aequorin, and Maaz and Stieve (1980), using arsenazo III, showed that the amplitude of the indicator response was related to the adaptational state of the cell. In those experiments, the state of adaptation was varied by changing the interstimulus interval for a pair of equally bright stimuli: the aequorin or the arsenazo signal elicited by the second stimulus

of the pair became smaller as the interstimulus interval decreased. However, the value of Ca_i before the second bright stimulus is not known. We show in Fig. 10 that it is the sensitivity and not Ca_i that seems to be related to the decrement of the Ca_i increase induced by the second bright stimulus. After the first bright flash, Ca_i was allowed to return to the dark level before a second bright flash of the same intensity was presented. During this time, sensitivity measured with test flashes had not returned to the prestimulus level. It can be seen in Fig. 10 that the second Ca_i transient is reduced in amplitude. When sensitivity fully recovered, so did the amplitude of the Ca_i transient (not shown). Thus, sensitivity, but not resting Ca_i, seems to be related to the magnitude of the Ca_i increase. Such a result might imply the existence of a relationship between the source of Ca ions and the sites that mediate sensitivity.

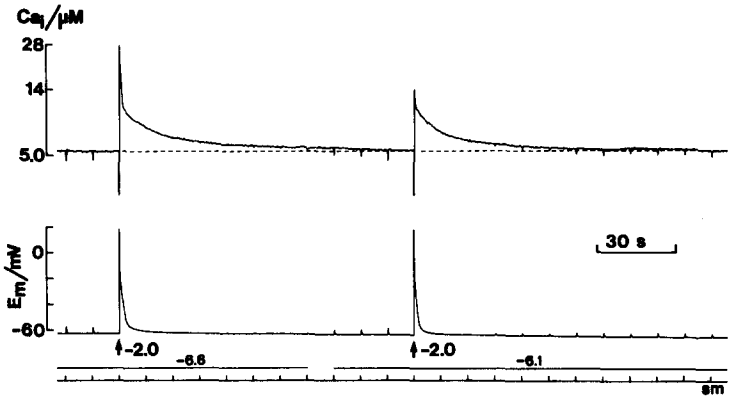

FIGURE 10. Light-induced Ca_i increase at different states of adaptation. Top trace: Ca signal. Middle trace: membrane potential. The sensitivity of the cell was monitored continuously with dim 20-ms-long flashes presented every 10 s, the intensities of which are shown by the numbers and bars above the stimulus monitor (sm). At the time marked by each arrow, a 20-ms bright flash of log intensity −2.0 was presented. Later (not illustrated for ease of presentation), sensitivity recovered fully to its prestimulus level.

Time Course of Ca_i and Dark Adaptation after a Bright Flash

As shown in Fig. 10, Ca_i returned to the prestimulus level after the first bright flash, while sensitivity lagged behind by ~0.5 log units. Under these conditions, there appears to be an uncoupling between Ca_i and sensitivity. Where it was examined, the time course of dark adaptation after a bright flash was found to be consistently slower than the recovery of Ca_i to the prestimulus level. In a given cell, it was found that the brighter the stimulus, the longer it took for Ca_i and sensitivity to recover to their prestimulus levels. Depending on the intensity of the stimulus (range −1.0 to −3.5), the time for sensitivity to recover to its prestimulus level was longer than the recovery of Ca_i by a factor of 1.2–2.4. The time course of Ca_i after a bright flash can sometimes be complex; we noticed that in cells having S_D values of <4.6, Ca_i can actually undershoot the prestimulus

level after a light flash, during a time when sensitivity is still depressed (Levy, 1982).

Effect of EGTA Injection on Ca_i and Sensitivity

A necessary corollary of the Ca hypothesis for light adaptation is that reducing the light-induced Ca_i increase should reduce the associated desensitization and thereby shorten the time course of dark adaptation. One manipulation that apparently reduces the light-induced Ca_i increase is the intracellular injection of the Ca chelator EGTA (J. E. Brown and Blinks, 1974; Lisman and Brown, 1975*b*). Lisman and Brown (1975*b*) reported that in addition to its expected effects, injection of EGTA unexpectedly desensitized *Limulus* ventral photoreceptors. It seemed important to determine whether or not this additional effect was mediated by a change in Ca_i. In addition, injection of a Ca chelator provides a means of showing that the Ca electrode indeed measures changes in Ca_i. Fig. 11 shows an experiment in which EGTA was injected by ionophoresis through the intracellular voltage electrode. Before injection, the cell was stimulated with brief adapting flashes of log relative intensity −2.3 (*A*) and −1.5 (*B*); these elicited, respectively, a relatively small and a relatively large Ca_i increase. Fig. 11*C* shows several typical effects of EGTA seen at 2 min after the injection: (*a*) an apparent decrease in sensitivity, as evidenced by a higher test flash intensity needed to produce the same amplitude criterion response as before the injection; (*b*) a resting Ca_i in the dark-adapted state of the same value or slightly increased relative to that before the injection; (*c*) a prolongation of the time to peak of responses to dim test flashes, as shown in the inset of Fig. 11; and (*d*) a much smaller light-induced Ca_i increase. The effect of an increase in buffering capacity (by injection of EGTA) on the light-induced Ca_i increase is similar to a decrease in light intensity (compare *A* and *C*). Fig. 11*F* shows that the effect of EGTA injection remains after 65 min. Fig. 11*E* shows that the decrease in the light-induced Ca_i increase is not due to a loss of sensitivity of the Ca electrode following the injection, since increasing the intensity of the stimulus by 1 log unit resulted in a bigger Ca_i increase (see Fig. 6).

The effect of EGTA injection on the latency of the photoresponse is in agreement with previous reports (Lisman and Brown, 1975*b*; Martinez and Srebro, 1976; J. E. Brown and Coles, 1979). However, it was previously thought that the time to peak (t_p) of the light response was strongly dependent on resting Ca_i (Martinez and Srebro, 1976; Lisman, 1976). We show here (Fig. 11 and Table V) that the increase in t_p after injection of EGTA does not result from a decrease in resting Ca_i. In contrast, we show in the next section that a decrease in resting Ca_i, resulting from a decrease in Ca_o, *is* associated with an increase in t_p. Thus, an increase in t_p cannot be construed to imply a decrease in Ca_i, although the converse may be true.

Table V gives a summary of the effects of EGTA injections in four photoreceptor cells. Immediately after injection, the resting Ca_i was usually close to its value preceding the injection, which suggests that Ca_i homeostasis was active during the injection time. However, in cell 1A of Table V, resting Ca_i was at a much lower value (~0.01 μM) immediately after the injection; Ca_i recovered to the control resting Ca_i in ~10 min (half-time 1 min). For this cell, the decrease

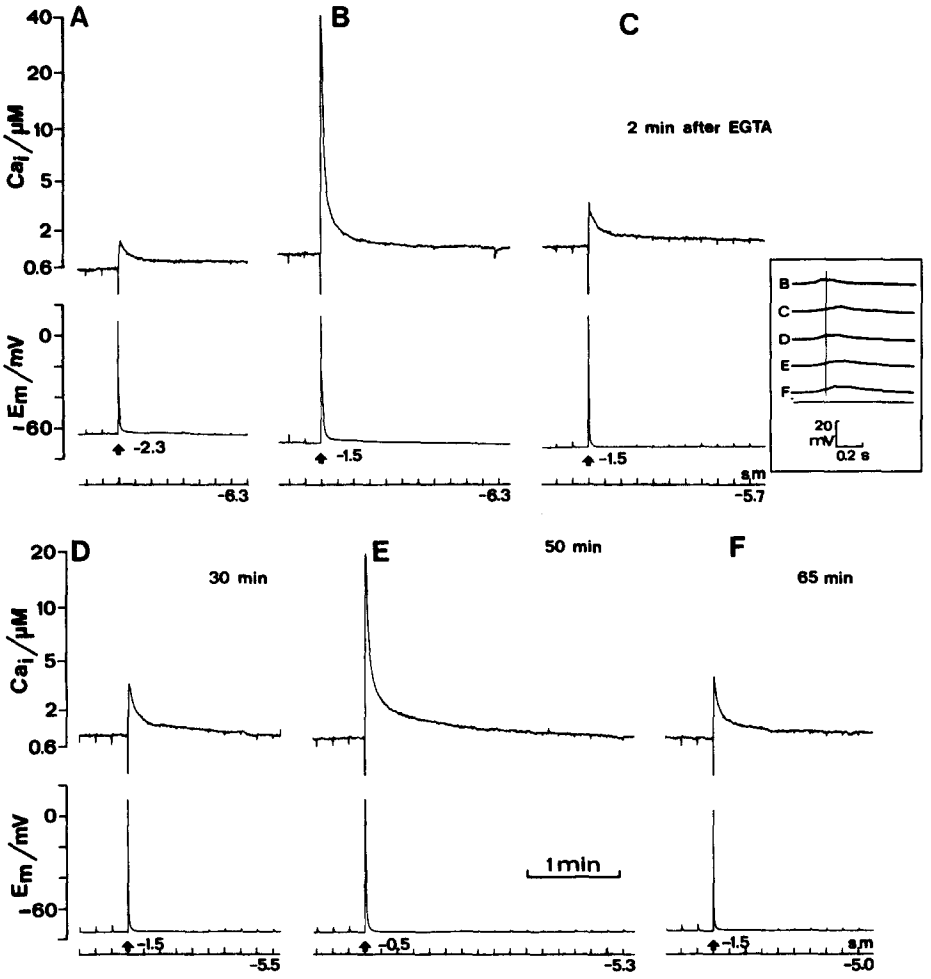

FIGURE 11. Effect of ionophoretic injection of EGTA upon Ca_i and sensitivity. Top trace: Ca signal. Middle trace: membrane potential. Lowest trace: stimulus monitor. Sensitivity was measured throughout by 20-ms test flashes whose log intensity is shown under the sm trace. The arrows and associated numbers indicate the time and log intensity of the 20-ms adapting flashes. (*A* and *B*) Light-induced Ca_i increases at two different intensities before injection. After trace *B*, the cell was allowed to dark-adapt before the injection was started. (*C*, *D*, *F*) Light-induced Ca_i increases at various times (as indicated) after an EGTA injection of 3.0 μC (5 nA for 10 min); the ΔCa_i elicited by a flash of same intensity as *B* is greatly reduced. (*E*) The stimulus intensity was increased by 1 log unit to show that the increase in Ca_i, as measured by the Ca microelectrode, was still graded with light intensity. The inset shows sample oscilloscope traces of responses to test flashes on an expanded time scale. The time of the stimulus is indicated by the deflection of the stimulus monitor (lowest trace). The intensity of each test flash corresponds to that indicated in each panel (*B*, *F*) of the figure. The responses were recorded right before the stimulus that elicited each respective Ca signal, except for response *B*, which was recorded before the beginning of the EGTA injection. After 65 min (*F*), the photoresponse is still prolonged and slowed.

in resting Ca_i immediately after the injection can be explained by the rapid influx of chelator: the injection was done by passing −10 nA for 3.3 min, as opposed to the other cells, where −3 or −5 nA for 10 or 13 min was used.

Sensitivity, as measured by a response of criterion amplitude, generally decreased by 0.4–0.6 log units after EGTA injection. However, since the photoresponse is more prolonged, its time integral might be a more representative measure of sensitivity. When this correction was made (see Methods and Table

TABLE V

Effect of EGTA Injection on Ca_i and Sensitivity

Cell	Injection	Time after injection	Ca_i^p	$\log(1/S_D)$	t_p	Corrected $\log(1/S_D)$*	Photostimulation: Log relative intensity‡	Photostimulation: ΔCa_i
	μC	*min*	*μM*		*ms*			*μM*
1A	2.0§	Control‖	2.73	−5.2	240	−5.2	−1.0	30.7
		4	1.76	−5.3	366	−5.26	−1.0	5.5
		18	2.85	−5.2	283	−5.1	−1.0	28.1
1B	3.0§	Control‖	2.80	−4.9	217	−4.9	−1.0	28.1
		3	2.80	−5.0	395	−5.3	−1.0	4.4
		19	3.31	−5.0	¶	¶	−1.0	10.2
2	3.0	Control‖	1.00	−6.1	256	−6.1	−1.5	40.6
		2	1.09	−5.7	411	−6.1	−1.5	2.1
		7	1.32	−5.6	306	−5.9	−1.5	2.3
		30	0.90	−5.4	320	−5.5	−1.5	2.3
3	2.4	Control‖	1.58	−6.3	308	−6.1	−1.5	11.2
		2	1.78	−5.7	494	−6.0	−1.5	1.9
		16	1.78	−5.8	337	−6.1	−1.5	1.8
4**	1.8	Control‖	0.93	−6.4	278	−6.4	−0.5	0.78
		3.5	0.97	−6.0	378	−6.3	−0.5	0.51
		41	0.85	−5.9	¶	¶	−0.5	0.41

* Corrected $\log(1/S_D) = \log(1/S_D) - \log$(area test flash after injection/area test flash control).

‡ Duration, 20 ms.

§ Two injections in the same cell.

‖ Before injection.

¶ Area of response not available.

** Photoreceptor impaled in a region away from the most sensitive area.

V), there was almost no change in sensitivity after the injection. Injections in cell 1 of Table V did not cause any decrease in sensitivity even before correction. The last column of Table V shows that injection of EGTA resulted in a dramatic decrease of the light-induced Ca_i increase (ΔCa_i) in all cells; the fractional change in ΔCa_i (ΔCa_i after injection/ΔCa_i control) varied between 0.05 and 0.2 in the first minutes after the injection. In some cells, ΔCa_i recovered somewhat with time (see Fig. 11 and Table V). A decrease in ΔCa_i was also measured away from the site of highest sensitivity (cell 4, Table V).

Effect of Low Ca_o on Ca_i and Sensitivity

Bathing *Limulus* ventral photoreceptors in low-Ca ASW (Ca_o) has been reported to increase both the sensitivity (Millecchia and Mauro, 1969*a*, *b*) and latency of the photoresponse (Martinez and Srebro, 1976; Lisman 1976). It was further reported that bathing the photoreceptors in low Ca_o for more than 10 min eventually leads to their desensitization (Millecchia and Mauro, 1969*a*, *b*). The reported increase in sensitivity and latency is probably mediated by a decrease in Ca_i, but what could account for the subsequent decrease in sensitivity? Beyond this question, a controversy exists regarding the source of the Ca ions that gives rise to the light-induced Ca_i increase (J. E. Brown and Blinks, 1974; Lisman, 1976; Maaz and Stieve, 1980). Here we present evidence that bathing the photoreceptors in low Ca_o leads to: (*a*) a simultaneous decrease in Ca_i and an increase in sensitivity during the first 10–15 min of exposure; (*b*) a decrease in sensitivity following a longer exposure to low Ca_o, even though Ca_i remains lower than in the control; (*c*) an increase in the latency of the response to a test flash in the dark-adapted state; and (*d*) a persistence of the light-induced rise in Ca_i in photoreceptors bathed in low Ca_o for up to 35 min.

Fig. 12*A* shows a recording of Ca_i and sensitivity during a change of $[Ca^{2+}]_o$ from 10 to 0 mM (a Ca_o lower than 1 nM was measured). 0 Ca_o caused a simultaneous decrease in Ca_i and an increase in sensitivity. Typically, sensitivity stabilized with a half-time of ~1 min, whereas it took Ca_i between 3 and 6 min (half-time). It was found that the changes were slower if EGTA was not added to the superfusate (see Table VI). In the dark-adapted state, the effect of a short exposure to low Ca_o on Ca_i and sensitivity was variable. Table VI shows that in all photoreceptors Ca_i decreased, whereas sensitivity did not always increase at these times. The relative decrease in Ca_i (Ca_i in low Ca_o/Ca_i control) varied from 0.1 to 0.7 after ~10 min of superfusion in low Ca_o. In most cells, the associated increase in sensitivity varied from 0.2 to 0.8 log units.

We found that the cells eventually desensitized when the exposure to low Ca_o was prolonged. During the same time, however, Ca_i either remained constant or decreased even further. In cells 5 and 6 of Table VI, even though the measure of sensitivity was corrected for the increase in the time integral of the responses, sensitivity still did not increase as Ca_i decreased. In other words, a decrease in Ca_i was associated with a relative desensitization of the cell. This indicates that, at least under certain conditions, a variation in Ca_i is not always coupled to a corresponding change in sensitivity.

Low-Ca_o exposure increased both the time to peak and the duration of the response, as shown previously (Lisman, 1976; Martinez and Srebro, 1976). As a result, the time integral of the response increased two- to threefold. Thus, an increase in t_p seems to correlate well with the decrease in Ca_i.

Fig. 12*B* shows the effect of lowering Ca_o on the light-induced Ca_i increase. After 5 min in 0 Ca, the cell had depolarized and lost sensitivity. A bright flash of the same intensity as the control elicited a ΔCa_i of about half the size of the control. In most cells, the ΔCa_i was either equal to or smaller than that of the control; however, because Ca_i had decreased, the relative increase in Ca_i induced by light was actually bigger (see Table VI). In none of the cells bathed in low

Ca_o could we see a disappearance of the light-induced Ca_i increase. In one cell, repetitive light stimulation for 35 min in 0.1 mM Ca ASW led to a decrease but not to a suppression of the Ca_i increase. Because prolonged exposure to low Ca_o

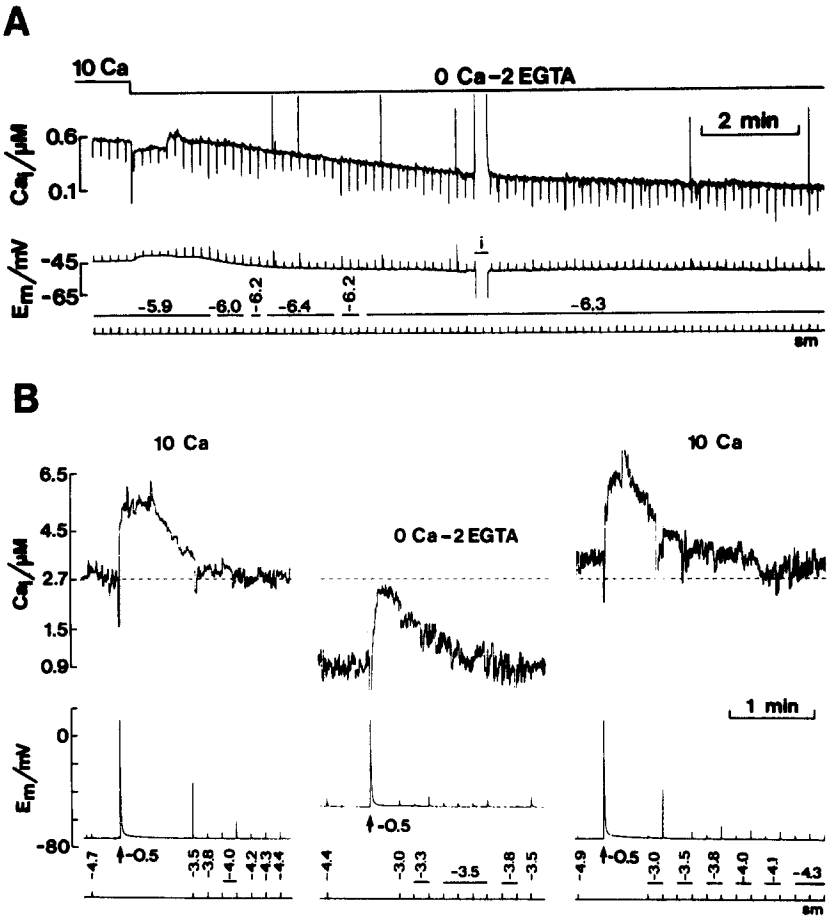

FIGURE 12. Effect of low Ca_o on resting Ca_i, sensitivity, and ΔCa_i. Top trace: Ca signal. Middle trace: membrane potential. Lower trace: stimulus monitor. The sensitivity of the cell was monitored continuously with 20-ms test flashes repeated every 10 s, whose log intensities are given by the bars and numbers over the sm trace. (*A*) Changing the superfusate from 10-Ca ASW to 0-Ca ASW caused a simultaneous decrease in Ca_i and an increase in sensitivity. The increase in sensitivity is evidenced by the fact that the criterion response is obtained at a lower log relative intensity. The change in solution produced a transient electrical artifact on both traces. *i* denotes the time when 1 nA was passed through the voltage electrode to measure the input resistance. Cell 5 of Table VI. (*B*) Effect of low Ca_o on the light-induced Ca_i increase. Light-evoked Ca_i increase before (left) and after (right) changing the superfusate to low Ca_o. Middle: after 5 min in 0 Ca. The time and intensity for each 20-ms-long stimulating flash is indicated by arrows. The dashed line indicates the level of Ca_i in the dark before changing to low Ca_o.

TABLE VI

Effect of Low Ca_o on Ca_i and Sensitivity

Cell	Perfusate	Time exposure	Ca_i^P	$\log(1/S_D)$	Corrected $\log(1/S_D)$*	Log relative intensity	Photostimulation	
							Duration	Ca_i^L/Ca_i^P (peak)
		min	*μM*				*s*	
1	ASW	Control‡	1.22	−6.2	—	−0.6	0.02	9.0
	0.1 Ca	5	0.89	−6.4	NA	"	"	12.6
	"	35	0.44	−5.7	NA	"	"	14.5
	ASW	Control§	2.70	−6.6	—	"	"	3.7
2	ASW	Control‡	2.53	−4.9	—	−0.8	190	1.3
	0 Ca	5	1.22	−4.6	NA	−0.5	109	1.3
	"	35	1.25	−4.8	NA	—	—	NA
	ASW	Control§	4.35	−5.0	—	—	—	NA
3	ASW	Control‡	2.70	−4.6	—	−0.5	0.02	2.0
	0 Ca	5	0.91	−4.4	NA	"	"	2.6
	"	13	0.91	−3.9	NA	—	—	NA
	ASW	Control§	3.10	−4.9	—	−0.5	0.02	2.1
4	ASW	Control‡	6.40	−5.0	—	0.0	116	1.6
	0 Ca	6	2.80	−5.3	NA	"	166	Off scale
	"	20	2.30	−4.3	NA	—	—	NA
	ASW	Control§	10.10	−4.8	—	—	—	NA
5	ASW	Control‡	0.56	−5.9	−5.9	−5.0	180	3.5
	0 Ca	15	0.09	−6.3	−6.7	"	"	22.9
	"	35	0.01	−5.1	−5.8	—	—	NA
	ASW	Control§	NA	NA	—	—	—	NA
6	ASW	Control‡	1.25	−5.4	−5.4	−1.5	0.02	3.3
	0 Ca	8	0.22	−5.8	−6.2	"	"	10.1
	"	28	0.16	−5.2	−5.8	—	—	NA
	ASW	Control§	1.00	−5.4	−5.4	−1.5	0.02	7.4

* Corrected $\log(1/S_D) = \log(1/S_D) - \log$(area test flash after exposure Ca_o/area test flash control).

‡ Before.

§ After.

NA, not available.

also desensitized the cell, the decrease in ΔCa_i seen could be due to the possible effect that sensitivity has on ΔCa_i (see Fig. 10).

Upon returning to normal ASW, most cells gained Ca^{2+}, but this was not always associated with a concomitant decrease in sensitivity.

DISCUSSION

The Resting Ca_i

The mean resting Ca_i was 3.5 μM ± 2.5 SD for 31 photoreceptors having a log threshold less than or equal to −4.6 (see Fig. 4*A*). This value is about one order of magnitude higher than that reported for other neurons, using Ca microelectrodes (H. M. Brown and Owen,1979; Alvarez-Leefmans et al., 1981; Gorman et al., 1984). One possibility is that the high resting Ca_i might result from damaged cells that had a reduced resting membrane potential (Alvarez-Leefmans et al., 1981). Our findings do not support this notion, as there was no apparent

relationship between resting Ca_i and E_m. Moreover, we observed that the photoreceptors can depolarize or hyperpolarize by as much as 20–30 mV without noticeably affecting the value of the resting Ca_i. A better indicator of photoreceptor function is the cells' dark-adapted sensitivity (S_D). As shown in Fig. 4*A*, there was no obvious correlation between Ca_i and S_D. Furthermore, some cells had lost all sensitivity to light during impalement by the Ca electrode but still had a resting Ca_i close to or even lower than 1 μM. On the other hand, Fig. 4*B* shows that there is perhaps a weak relationship between Ca_i and the membrane input resistance (R_i). Resting Ca_i does not appear to be directly related to the three different indicators of cell damage, E_m, S_D, and R_i.

What other factors might give rise to an artificially high Ca_i? One possible source of error could be the fact that the Ca signal, $E_{Ca} - E_m$, is a differential measurement (see Methods and Fig. 3). In the unlikely instance where the E_m measured by the V electrode is systematically 10 mV larger than the one measured by the Ca electrode, the resting Ca_i would be 0.3 μM instead of 0.1 μM (as shown in Fig. 2*C*, a change of 10 mV will decrease the pCa from 7 to 6.5). An elevated resting Ca_i might be due to loading of Ca^{2+} from the superfusate or because of local membrane leakage around the Ca electrode. The former is unlikely because *Limulus* hemolymph was actually found to have a higher Ca^{2+} concentration (~13 mM) than the ASW used.

The latter possibility of a defective membrane seal around the Ca electrode seems unlikely because decreasing Ca_o by several orders of magnitude (from 10 mM to nominally 0) leads to only a small decrease in Ca_i with a half-time of 3–6 min, while the chamber volume was being exchanged three times per minute (see Fig. 12). This Ca_i decrease was seen in all cells, regardless of their resting Ca_i (Table VI); it was also measured with aequorin (Bolsover and Brown, 1982).

Limulus ventral photoreceptors may have a requirement for a higher Ca_i than encountered in other neurons. Superfusion with 0-Ca ASW for >15 min leads to a decrease of Ca_i (see Table VI) and of the light response (Millecchia and Mauro, 1969*a*, *b*); injection of Ca^{2+} in such depleted cells leads, surprisingly, to an increase of the light response (Bolsover and Brown, 1982). This could indicate that Ca ions are required in order for phototransduction to proceed. However, it is not a direct indication that the resting Ca_i is as high as measured here, and our results should therefore be considered an upper limit.

The Spatial Localization of the Light-induced Ca_i Increase

The results presented in Table II and Fig. 5 indicate that the light-induced Ca_i increase is on the average ~20 times faster and larger in the most sensitive region of the cell than away from it. Using arsenazo III, Harary and Brown (1984) had shown that the light-induced Ca_i increase was spatially nonuniform, but they did not indicate whether the increase was localized to a particular region of the photoreceptor. Stern et al. (1982) had shown that light adaptation was probably initiated in the R lobe (the region of highest sensitivity). We suggest that both light adaptation and the Ca_i increase are initiated in the R lobe.

Some cells had an almost uniform sensitivity profile, and the light-induced Ca_i increase in these cells could either be small or large (Table II). Since the

illumination in these experiments is passing through the photoreceptors from below, the uniform sensitivity profile would occur whether the R lobe is located on top of or under the cell. However, the location of the R lobe will determine whether the Ca electrode will enter the R or A lobe, and hence will measure a large or small Ca_i rise.

The small increase in Ca_i ~60 μm away from the most sensitive region after uniform illumination could originate either from a local Ca_i increase or from diffusion from the most sensitive region. We cannot rule out the possibility that the plasma membrane of the A lobe contains rhodopsin, which can given rise to a local Ca_i increase upon photon absorption. However, if the small Ca_i increase at a distance is the result of diffusion of Ca^{2+} from the most sensitive region, then it should be possible to calculate its amplitude using diffusion equations that approximate the experimental conditions. In most cases, the R lobe occupies one end of the cell (see Figs. 1 and 5, *A* and *B*). Therefore, we can assume that initially the Ca_i increase is concentrated in a finite region comparable to the extent of the R lobe. Since this region is limited on one side by the plasma membrane, this situation can be approximated by a one-dimensional semi-infinite diffusion problem whose solution is given by (Crank, 1956, p. 13):

$$C(x) = \tfrac{1}{2}C_o\left[erf\frac{(h-x)}{2(Dt)^{1/2}} + erf\frac{(h+x)}{2(Dt)^{1/2}}\right] \quad (3)$$

where C is the concentration of Ca^{2+} having an initial concentration distribution at $t = 0$ of $C = C_o$ for $0 < x < h$, $C = 0$ for $x > h$; *erf* is the error function; D is the effective diffusion coefficient for Ca^{2+} in the cytosol, ~10^{-7} cm^2/s (Kushmerick and Podolsky, 1969; Gorman and Thomas, 1980; Fein and Charlton, 1978*a*); and t is the time for Ca^{2+} to reach a concentration C at a distance x.

From the results summarized in Table II, we can assume that Ca_i increases by ~30 μM (C_o) throughout the R lobe, which we can take (see Fig. 1) to have an extent of ~30 μm (h). Ca_i typically reaches its maximum in 20 s, at a distance 60 μm away from the R lobe (see Table II). Using these figures, the calculated Ca_i is ~2 μM, which is close to the average value of 1.5 μM measured experimentally (Table II, *A*).

The Magnitude of the Light-induced Ca_i Increase

When measured in the R lobe, Ca_i reaches concentrations of ~30–40 μM after a flash (Figs. 3, 5*B*, and 6). This should be considered a lower limit because of the response time of the Ca microelectrode. Using arsenazo III, Brown et al. (1977) estimated that Ca_i reached a level of ~100 μM during illumination of the same photoreceptor. By comparison, voltage-clamp stimulation of *Aplysia* neurons increases Ca_i by only 1 μM or so, as measured by Ca-sensitive microelectrodes (Gorman et al., 1984), whereas in honeybee drone photoreceptors, Ca_i increases by ~10 μM during photostimulation (Levy, 1979).

The Relationship Between the Light-induced Ca_i Increase and Sensitivity

It has been shown previously that injection of Ca ions into *Limulus* ventral photoreceptors can mimic the action of an adapting light in its ability to desen-

sitize the cells (Lisman and Brown, 1972*b*, 1975*b*; Fein and Charlton, 1977*a*, 1978*a*). However, for Ca^{2+} to mediate light adaptation, its rise should be graded over the entire range that adaptation occurs (e.g., Fein and Szuts, 1982). In Fig. 9, we show that the increase in Ca_i is related to the decrease in sensitivity, starting from the threshold of adaptation and extending over several log units of desensitization. The two bear the following power relationship: $(Ca_i^L/Ca_i^D)^{1/m} = S_D/S_L$, where the mean value of the slope m, when both parameters are plotted on log-log coordinates, is 0.20 (range 0.07–0.26; see Table IV). Such a low value for m indicates a remarkably steep relationship: a 100-fold reduction in sensitivity is associated with an ~2.5-fold steady increase in Ca_i. This steepness is not unique to the relationship between sensitivity and Ca_i. For example, Fuortes and Hodgkin (1964) found that a 200-fold decrease in sensitivity was associated with a halving of the latency of the light response in *Limulus* lateral eye photoreceptors. Also, Fein and Charlton (1977*a*) reported that a 50-fold change in sensitivity was associated with a 2-fold change in the photoresponse time delay for ventral photoreceptors.

What are the reasons for such a steep relationship? Some limitations on the steady Ca_i increase seem obvious. Ca_i cannot possibly cover ~4 log units (see Fig. 9) as adaptation does, since it would have to increase from, say, 1 μM to 10 mM, which, a priori, appears to be impossible. Large increases in I_b might induce only small increases in Ca_i, but might induce large increases in threshold for several reasons. First, as I_b increases, the increase in Ca_i slows down, because as Ca^{2+} accumulates near the membrane, its driving force decreases (Gorman and Thomas, 1980; Fischmeister and Horackova, 1983). Ca^{2+} accumulates more easily than Na^+ or K^+ because it diffuses ~100 times more slowly. Second, as I_b increases, the increase in Ca^{2+} load could trigger recruitment of other buffers (for example, mitochondria; see Brinley, 1978). Third, as I_b increases, sensitivity decreases; this in turn may reduce the ΔCa_i per photon, which comprises a negative feedback mechanism. Fig. 10 shows that the adaptational state of the cell seems to be related to the magnitude of the light-induced Ca_i increase, in accordance with previous reports (J. E. Brown and Blinks, 1974; Maaz and Stieve, 1980). Finally, small increments of Ca_i might induce large desensitizations if several reactions of transduction are modulated by Ca^{2+} during light adaptation. From the average value of m in Table IV, desensitization seems to be equal to the fifth power of Ca_i. The location of the Ca microelectrode within a few microns from the site of Ca^{2+} increase cannot account for the steepness of the relationship because the duration of each background light was long enough to allow equilibration of Ca_i within a radius of a few microns (Fischmeister and Horackova, 1983).

For the cell of Fig. 7, where the resolution of the Ca signal was good enough to allow the measurement of an increase in Ca_i associated with a desensitization of 0.1 log unit, we estimate that 10^3–10^4 Ca ions are released per photoactivated rhodopsin within 60 s (assuming the volume of the R lobe to be 80 pl). This is very similar to what might be released from a vertebrate rod disk by one photon at threshold (Hagins and Yoshikami, 1974).

Comparison with Previous Measurements of Ca_i

If the light-induced rise in Ca_i initiates light adaptation, then it follows that the Ca_i rise should precede the desensitization. We were unable to demonstrate this in our measurements. J. E. Brown and Blinks (1974) have shown that at high intensities, the peak of the aequorin signal occurs during the decline of the light response from transient to plateau, which is when desensitization begins to occur (Lisman and Brown, 1975*a*). On the other hand, they were unable to detect a sustained aequorin luminescence during a prolonged bright stimulus, which contrasts with the results of this study. The discrepancy might arise because the rate of light emission by aequorin is not linearly related to free Ca concentration changes, as discussed by Gorman and Thomas (1980) and Blinks et al. (1982). However, the decay time of Ca_i changes measured with the Ca microelectrodes resemble those measured by arsenazo III in the same preparation (J. E. Brown et al., 1977; Nagy and Stieve, 1983).

Modification of the Relationship Between Ca_i and Sensitivity

In this section, we consider the validity of the relationship under altered conditions. For example, are variations in Ca_i always associated with variations in sensitivity in the right direction? Conversely, are variations in sensitivity always associated with changes in Ca_i?

TIME COURSE OF Ca_i AND SENSITIVITY AFTER A BRIGHT FLASH Fig. 10 shows that after the transient increase in Ca_i induced by a bright flash, Ca_i recovers to the prestimulus level at a time when sensitivity is still depressed, typically by 0.5–0.6 log units. On the other hand, with brighter stimuli of longer durations, the difference in recovery is less striking (see Fig. 7). In view of the direct relationship between the light-induced increase in Ca_i and desensitization during steady light, the apparent uncoupling between the time course of dark adaptation and Ca_i after a bright flash seems puzzling. It is possible that the discrepancy arises from the different stimulus conditions in the two situations. The relationship in question was determined by measuring Ca_i and sensitivity during the steady state induced by long steps of light: under these conditions, it is reasonable to assume that Ca_i and the sites that presumably bind Ca^{2+} and thereby control sensitivity had reached an equilibrium. The situation is quite different when a single brief flash of light elicits a large and transient increase in Ca_i that transiently depresses sensitivity. In this case, one would expect Ca_i and the sites controlling sensitivity not to reach equilibrium; Ca_i might then be returned to its prestimulus level by the buffering system, while sensitivity would return more slowly to the dark level; hence the uncoupling in time course. The amount of Ca bound to the sites would correlate with sensitivity, even though Ca_i does not (also see Lisman and Brown, 1975*b*). However, with brighter and longer stimuli (Fig. 7), more Ca^{2+} will accumulate that would take more time to be cleared away because of its low diffusion coefficient. The experimental result of Fig. 10 shows that after a bright flash, Ca_i does not seem to be the rate-limiting step in the final phase of dark adaptation, in agreement with Nagy and Stieve (1983). We suggest that the

release of Ca ions from the sites that control sensitivity is the rate-limiting step. However, we cannot exclude other alternatives such as a region inaccessible to the Ca electrode where the time course of Ca_i matches that of sensitivity during dark adaptation.

EFFECT OF EGTA INJECTION ON Ca_i AND SENSITIVITY The injection of EGTA into *Limulus* photoreceptors has been shown to decrease the light-induced sensitivity changes (Lisman and Brown, 1975*b*). J. E. Brown and Blinks (1974) had shown that injection of EGTA diminishes or even suppresses the aequorin signal, which is normally induced by a bright stimulus. The results presented in Fig. 11 and Table V are consistent with these reports. By simultaneously measuring sensitivity and Ca_i, we show that injection of EGTA results in a faster recovery of sensitivity after a bright flash. In that respect, EGTA has an effect similar to a decrease in light intensity: injecting ~3 μC of EGTA has the same effect on the light-induced Ca_i increase and on the recovery of sensitivity as reducing the light stimulus by ~1 log unit.

Lisman and Brown (1975*b*) reported that injection of EGTA resulted in a loss of absolute sensitivity and in an increase of the latency of the light response. Both effects could result from a change in resting Ca_i, but not in the same direction. A loss of absolute sensitivity should be due to an increase in Ca_i, whereas an increase in latency can be caused by a decrease in Ca_i (Lisman, 1976; see also low-Ca_o effects in the Results). We have shown that injection of up to 3.0 μC of EGTA does not alter resting Ca_i, and, when correction is made for the increase in time integral of the response, no decrease of absolute sensitivity is seen (Table V). There is thus no uncoupling between Ca_i and sensitivity for injections of up to 3 μC of EGTA. It is not surprising that injection of a Ca-chelating agent does not alter the resting Ca_i if it is assumed that Ca_i homeostasis is set by the plasma membrane pump/leak system, as discussed by Tsien et al. (1982). Injection of a Ca buffer results in a net entry of Ca^{2+} from the external medium, with the overall effect that the total Ca is increased, whereas Ca_i remains unchanged. Evidence for this mechanism was shown in intact lymphocytes by Tsien et al. (1982); loading the cells with millimolar amounts of a Ca chelator in 0-Ca external medium resulted in a large decrease in Ca_i, which reversed rapidly to the preloading value when Ca^{2+} was added in the external medium. Homeostasis of Ca_i seem to be equally effective in *Limulus* ventral photoreceptors during injection of EGTA.

TIME TO PEAK OF THE LIGHT RESPONSE: A SPECULATIVE MODEL If the resting Ca_i does not decrease after injection of EGTA, what then could account for the increase in latency of the light response? Until now, the consensus was that the time to peak (t_p) of the light response was related to the resting Ca_i.

Payne and Fein (1983) have proposed that it is the increase in buffering capacity that is responsible for the increase in t_p, when the cell is injected with EGTA. If we assume that there are intracellular regulatory sites that control the t_p of the light response (Lisman, 1976) upon binding Ca, then an increase in the capacity for binding Ca^{2+} will decrease the number of Ca ions that reach the regulatory sites. Therefore, we suggest that it is both the variation in this capacity for binding Ca^{2+} and the variation in resting Ca_i that mediate the change in t_p when the cell is light-adapted or bathed in 0-Ca ASW. During light adaptation

(or after Ca^{2+} injection), most of the Ca_i rise will be buffered and the overall result will be an increase in Ca_i and a decrease in the capacity for binding Ca^{2+}. When Ca_i decreases during 0-Ca ASW superfusion, the capacity for binding Ca^{2+} increases, which in turn increases the t_p.

EFFECT OF LOW Ca_o ON Ca_i AND SENSITIVITY Removal of Ca ions from the solution bathing *Limulus* ventral photoreceptors causes (Fig. 12): (*a*) a simultaneous decrease in Ca_i and increase in sensitivity to light in the first 10–15 min; (*b*) an increase in the time to peak of the response in the dark-adapted state; (*c*) a decrease in sensitivity to light after a longer exposure to low Ca_o, with Ca_i remaining lower than the control; and (*d*) a persistence of the light-induced Ca_i increase.

In low Ca_o, the decrease in Ca_i is readily explained by the removal of the large inward electrochemical gradient for Ca^{2+}, which normally causes Ca^{2+} to leak continuously into the cell. When the exposure to low Ca_o is brief, the decrease in Ca_i is associated with an increase of sensitivity to light (see Table VI). This may indicate that in 10 mM Ca ASW, the level of resting Ca_i has an inhibitory effect on the sensitivity to light.

Two observations remain unexplained. First, we showed that the resting Ca_i does not seem to be related to the absolute sensitivity in different photoreceptors (Fig. 4*A*). Second, with prolonged superfusion in 0-Ca ASW, Ca_i continues to decrease while sensitivity decreases (Table VI). This might be explained if Ca^{2+} were required in more than one step in the transduction process (Bolsover and Brown, 1982) and if some of the reactions were inhibited by Ca^{2+} (thereby initiating light adaptation), whereas others required Ca^{2+}. Then decreasing Ca_i below a certain level would remove some of the inhibition, whereas decreasing Ca_i even further might inhibit the reactions that require Ca^{2+}. In this vein, Bolsover and Brown (1982) found that injecting Ca^{2+} increased the light response of cells that were bathed for a long time in 0-Ca ASW.

Some ambiguity exists regarding the source of the light-induced Ca_i increase that mediates light adaptation in *Limulus* ventral photoreceptors. After a bright flash, Ca^{2+} could come either from intracellular stores or from the extracellular medium, which normally contains 10 mM free Ca^{2+}. Our findings (Fig. 12 and Table VI) seem to suggest that Ca^{2+} comes mainly from intracellular stores, since a bright light still induces a Ca_i rise in photoreceptors bathed in 0 Ca_o, in accordance with Brown and Blinks (1974) and in disagreement with Maaz and Stieve (1980). These results are also consistent with the observation that light adaptation still occurs in 0-Ca ASW (Lisman, 1976). Likely stores of Ca^{2+} have been identified in this and other invertebrate photoreceptors (Walz, 1979; Walz and Fein, 1983; Skalska-Rakowska and Baumgartner,1985).

The decrease in the light-induced Ca_i rise during an extended exposure to low Ca_o might result from the associated decrease in sensitivity. As shown in Fig. 10, the light-induced desensitization may contribute to the decrease of the Ca_i rise induced by a given light stimulus.

In conclusion, our results indicate that an important aspect of the Ca hypothesis for light adaptation in *Limulus* ventral photoreceptors is fulfilled; namely, that starting near the threshold of adaptation, steady Ca_i increases are associated with steady desensitizations over several log units of light intensity.

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