

Changes in Intracellular Free Calcium Concentration during Illumination of Invertebrate Photoreceptors

Detection with Aequorin

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ABSTRACT Aequorin, which luminesces in the presence of calcium, was injected into photoreceptor cells of *Limulus* ventral eye. A bright light stimulus elicited a large increase in aequorin luminescence, the aequorin response, indicating a rise of intracellular calcium ion concentration, Ca_i . The aequorin response reached a maximum after the peak of the electrical response of the photoreceptor, decayed during a prolonged stimulus, and returned to an undetectable level in the dark. Reduction of Ca_o reduced the amplitude of the aequorin response by a factor no greater than 3. Raising Ca_o increased the amplitude of the aequorin response. The aequorin response became smaller when membrane voltage was clamped to successively more positive values. These results indicate that the stimulus-induced rise of Ca_i may be due in part to a light-induced influx of Ca and in part to release of Ca from an intracellular store. Our findings are consistent with the hypothesis that a rise in Ca_i is a step in the sequence of events underlying light-adaptation in *Limulus* ventral photoreceptors. Aequorin was also injected into photoreceptors of *Balanus*. The aequorin responses were similar to those recorded from *Limulus* cells in all but two ways: (a) A large sustained aequorin luminescence was measured during a prolonged stimulus, and (b) removal of extracellular calcium reduced the aequorin response to an undetectable level.

INTRODUCTION

Changes in the concentration of intracellular free calcium (Ca_i) during and after illumination have been proposed to play important roles in the physiology of photoreceptors. For vertebrate photoreceptor cells it has been sug-

gested that the absorption of light leads to a rise in intracellular free calcium concentration and that this rise in Ca_i is a step in the sequence of excitatory events leading to the generation of the receptor potential (Yoshikami and Hagins, 1971; Hagins, 1972). For invertebrate photoreceptors (in particular for the ventral eye of *Limulus*), an increase in Ca_i may be a step in the sequence of events of light adaptation (Lisman and Brown, 1972, and footnotes 1 and 2). The previous evidence that light elicits a rise in Ca_i in both vertebrate and invertebrate photoreceptors was indirect. In this study we have attempted to detect directly changes of Ca_i in the photoreceptors of two invertebrate species.

Photoreceptor cells of the ventral eye of *Limulus* have a transmembrane potential of 45–60 mV (inside negative). Illumination elicits a positive-going change in membrane voltage, the receptor potential. With bright and prolonged illumination the receptor potential has a large initial transient phase which declines rapidly to a plateau phase that is maintained for the duration of the light stimulus. The total membrane current generating the receptor potential can be measured with a voltage-clamp technique (Millecchia and Mauro, 1969 *b*; Lisman and Brown, 1971 *a*). The membrane current induced by the stimulus light also has an initial transient phase which declines to a plateau during a prolonged stimulus. This waveshape is a reflection of the process of light adaptation; the change in membrane conductance per absorbed photon (which we refer to as the sensitivity) is largest during the transient phase and becomes smaller during the plateau phase (Lisman and Brown).¹

The evidence that intracellular calcium is involved in the process of light adaptation is as follows: (*a*) Intracellular iontophoretic injection of calcium ions leads to a reduction of the responsiveness of the photoreceptor (Lisman and Brown, 1972). (*b*) A solution which contains calcium plus EGTA can buffer free calcium concentration. Intracellular pressure injection of a Ca-EGTA solution tends to stabilize the sensitivity of the photoreceptor. The stabilized value of sensitivity after the injection of a particular Ca-EGTA solution varies with the concentration at which calcium is buffered in the injection solution; for higher free calcium concentrations the sensitivity is lower. Also, after injection of a Ca-EGTA solution, the waveshape of the response to light is altered. The transient and plateau phases become more nearly the same height; i.e., the response tends to become “square” (Lisman and Brown, 1971 *b*, and footnote 2). These results indicate that sensitivity is reduced when Ca_i is increased and suggest that Ca_i may increase during a response to light.

¹ Lisman, J. E., and J. E. Brown. Light-induced changes in conductance-increase per photon in *Limulus* ventral photoreceptors. Manuscript in preparation.

² Lisman, J. E., and J. E. Brown. The effects of intracellular injection of EGTA buffers on light-adaptation in *Limulus* ventral photoreceptors. Manuscript in preparation.

Because previous evidence for light-induced changes in Ca_i was indirect, we attempted to detect such changes with the calcium-sensitive luminescent protein aequorin (Shimomura et al., 1962, 1963). Aequorin, which is a family of closely related proteins (Blinks, 1971), emits light in the presence of low concentrations of calcium ions. The reaction is not specific for calcium, but calcium is the only substance likely to be found in living cells in sufficient concentration to activate the reaction (Shimomura and Johnson, 1973). Magnesium ions inhibit the reaction (van Leeuwen and Blinks, 1969; Blinks, 1973). The reaction sequence involves several steps, at least one of which is irreversible (Hastings et al., 1969). In vitro, the intensity of light emission increases steeply with increasing calcium concentration between 10^{-7} and 10^{-5} M, and is maximal at concentrations above 10^{-4} M (Shimomura et al., 1962; Blinks, 1973).

Aequorin has been used successfully to detect changes in Ca_i in a variety of giant cells (Ashley and Ridgway, 1970; Baker et al., 1971; Llinás et al., 1972; Stinnakre and Tauc, 1973). In this study, we have pressure-injected solutions of aequorin into invertebrate photoreceptors and have confirmed that changes in Ca_i are induced by light. A preliminary account of some of our results has already been presented (Brown and Blinks, 1972).

METHODS

Ventral rudimentary eyes of *Limulus polyphemus* (Demoll, 1914; Clark et al., 1969) or lateral ocelli of the barnacle, *Balanus eburneus*, (Fales, 1928; Brown et al., 1970) were mounted in a small Sylgard 184 (Dow Corning Corp., Midland, Mich.) chamber. Methods for recording, stimulating, and perfusing were as reported previously (Lisman and Brown, 1971 a, 1972) except as noted below. Artificial seawater (ASW) contained 423.0 mM NaCl, 9.0 mM KCl, 9.3 mM $CaCl_2$, 22.9 mM $MgCl_2$, 25.5 mM $MgSO_4$, 2.15 mM $NaHCO_3$, and 15 mM Tris-Cl adjusted to pH 7.8. Low calcium seawater (low Ca-SW) had 99% of the $CaCl_2$ replaced isosmotically with sucrose. The high calcium seawater (high Ca-SW) contained 46.4 mM $CaCl_2$ and the $MgSO_4$ was replaced by $MgCl_2$. In the low magnesium seawater (low Mg-SW) the magnesium salts were replaced isosmotically by Tris- SO_4 (at pH 7.8) or sucrose. In the solution containing no calcium or magnesium but having 5 mM EGTA, the divalent metal salts were replaced isosmotically by sucrose. All experiments were carried out at room temperature, which was about 22°C.

Aequorin extracted from specimens of the hydromedusan *Aequorea forskålea* (collected at Friday Harbor, Washington) was purified in 10 mM EDTA by a combination of gel filtration on G-50 Sephadex (fine) (Pharmacia, Piscataway, N. J.) and ion-exchange chromatography on A-50 DEAE Sephadex (each performed twice). The product was shown by isoelectric focusing to be a mixture of luminescent proteins with isoelectric points between pH 4.4 and 4.7. Polyacrylamide gel electrophoresis revealed no nonluminescent components. The purified aequorin was freed of EDTA by passage through a mixed bed of 90% G-25 Sephadex (medium) and 10% chelating resin (Chelex 100, 50–100 mesh, Bio-Rad Laboratories, Richmond,

Calif.) equilibrated and eluted with 10 mM ammonium acetate that had first been passed through a column of Chelex 100 in the ammonium form. The aequorin was then lyophilized in the presence of Chelex 100 beads. Since ammonium acetate sublimates during lyophilization, the product was salt free. For intracellular injection, the lyophilized aequorin was dissolved in 0.5 M KCl which had been freed of calcium by passage through a column of Chelex 100 in the potassium form. The concentration of active aequorin in the injection solution was assayed by measuring the total light yield of 10 μ l aliquots. Results were converted to molar concentration on the basis of an assumed quantum yield of 0.24 (Shimomura and Johnson, 1970). The solutions injected in these experiments had concentrations of active aequorin ranging from 0.1 to 0.5 mM.

Blunt micropipettes (with resistances of about 4 M Ω when filled with 3 M KCl) were filled with just enough aequorin solution to make contact with a platinum wire (silver inactivates aequorin; Blinks, unpublished finding). The solution was injected by applying pressure, 5–20 psi, to the back of the pipette. The electrode was usually left in place after the injection and was used to record changes of transmembrane potential. Because of the polarizable nature of the platinum wire in the recording electrode, it was not possible to measure a stable value for resting membrane potential over the duration of a typical experiment, which lasted several hours. When voltage-clamp studies were to be done, a second micropipette filled with 3 M KCl was introduced into the cell to measure membrane potential. Voltage-clamp current was passed through the electrode containing aequorin (in 0.5 M KCl).

Light emitted from the injected photoreceptor cell was collected with a microscope objective ($\times 20$, numerical aperture 0.4) and focused directly onto the photocathode of a 9635B (EMI-Gencom Div., Plainview, N. Y.) photomultiplier tube ("PMT") operated at 1,000 V. Stimulus light from a tungsten-iodide lamp was passed through heat filters, an electromechanical shutter (Vincent Associates, Rochester, N. Y.), and neutral density filters, and was focused on the photoreceptor. Stimulus intensity was calibrated with an Eppley thermopile (Eppley Laboratory, Inc., Newport, R. I.) or a Tektronix digital photometer (J 16) (Tektronix, Inc., Beaverton, Ore.).

Since the light stimuli were bright enough to produce prolonged changes in the performance of a PMT operated at high voltage, it was necessary to prevent the stimuli from reaching the photocathode. This was done in either of two ways. When brief flashes were used to stimulate the photoreceptor cell, an electromechanical shutter was used to prevent the stimulus from reaching the PMT; with this method (Figs. 1, 2, 4, 7, and 9) not all of the rising phase of the aequorin luminescence was recorded. When prolonged illumination was used as a stimulus, or when the rising phase of the luminescent response was to be observed, a mechanical chopper was used to interrupt the stimulus beam. A second mechanical chopper, locked 180° out of phase, was used to interrupt the light reaching the PMT. The two choppers were rotated fast enough to achieve "flicker fusion" of the photoreceptor response; in records made with this system (Figs. 3, 5, 8, 10, and 11) the aequorin luminescence appears repetitively chopped.

Transmembrane potential, voltage-clamp current, PMT anode current, and the timing of stimuli were recorded simultaneously on a magnetic tape recorder. Some figures for this paper were prepared by converting the analog records to digital data which were then scaled and plotted on an X-Y chart recorder.

In this paper concentrations of ions are denoted by chemical symbols and a subscript to indicate whether intracellular or extracellular is meant. For example, Ca_i is used instead of $[\text{Ca}^{++}]_{in}$. However, intracellular pH is denoted pH.

RESULTS

All of the experiments described in this paper were done on photoreceptors of both *Limulus* and *Balanus*. The results were similar except where specifically noted. The records for the figures shown were made from *Limulus* ventral photoreceptors, with the exception of Fig. 11. We have emphasized the results from *Limulus* ventral photoreceptors because previous experiments on these photoreceptors (Lisman and Brown, 1971 *b*, 1972) suggested that changes in Ca_i play a role in light adaptation.

The Aequorin Response

When a bright stimulus was applied to a *Limulus* or *Balanus* photoreceptor before the intracellular injection of aequorin, there was no luminescence or phosphorescence (Fig. 1 A). After a suitable amount of aequorin had been injected and the cell had remained in the dark for 2 min or more, a brief, bright stimulus elicited a large transient increase in luminescence (the "aequorin response") which significantly outlasted the stimulus (Fig. 1 A). Although the light emission was clearly due to the presence of aequorin inside the cell, the possibility remained that it was the result of phosphorescence rather than luminescence. Unreacted aequorin is not strongly fluorescent; aequorin that has reacted with calcium ("spent" aequorin) fluoresces much more intensely (Shimomura et al., 1963). Since no information about a long-lived phosphorescence of aequorin was available, we examined the possibility that spent aequorin might exhibit phosphorescence which could account for the aequorin response. Experiments carried out in an Aminco-Bowman (American Instrument Co. Silver Spring, Md.) spectrophotofluorometer showed that the decay of light emitted at 4,650 Å by spent aequorin was complete within the time (10 ms) required to operate the shutter on the exciting light (3,400 Å); there was no long-lived phosphorescence. Thus it seems certain that the aequorin response represents luminescence from the aequorin in the photoreceptor cell, and not phosphorescence from any part of the system.

Influence of Injection Volume

The aequorin response is influenced by the volume of aequorin solution injected. If a very small volume of the aequorin solution is injected, little or

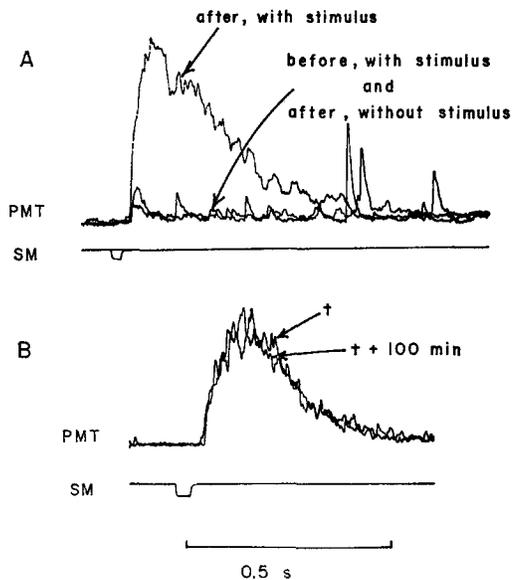


FIGURE 1

FIGURE 1. Aequorin responses measured from *Limulus* ventral photoreceptors. Photomultiplier current, PMT, (arbitrary scale) vs. time. The downward deflection of the stimulus monitor, SM, marks the duration of the stimulus light. A shutter in front of the PMT was opened just after the stimulus flash; therefore, not all of the rising edge of the aequorin response is recorded. (A) The three superimposed traces were recorded before injection of aequorin with the stimulus applied, and after injection, both without and with the stimulus. In the current trace measured before injection, the ambient light level is indicated by the slight increase recorded when the shutter opened. (B) Two aequorin responses elicited by identical stimuli; the second, slightly smaller response was elicited about 100 min after the first. In the interim, 15 additional stimuli were given.

FIGURE 2. Increase of aequorin responses and receptor potentials with time after injection of the aequorin solution. A shutter in front of the PMT was opened just after the stimulus flash. (A) Aequorin responses elicited by constant amplitude stimuli at 2-min intervals after injection. The time in minutes after injection is indicated for each trace. SM: stimulus monitor. PMT current in arbitrary units. (B) Superimposed receptor potentials, V, recorded simultaneously with records in A.

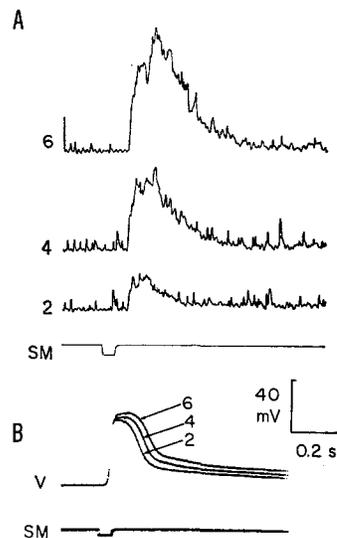


FIGURE 2

no aequorin response can be measured. If a larger volume is injected (with higher pressure or for a longer time), a typical aequorin response can be recorded (as in Fig. 1). If a much larger volume is injected, the receptor potential is diminished or abolished, and no transient change in aequorin luminescence can be observed after a brief, bright stimulus. After the injection of such a large volume, luminescence can be recorded from the unstimulated cell. This resting aequorin luminescence decays slowly (half-time of 15–20 min); also, the cell recovers its normal sensitivity to stimuli very slowly

(Fig. 5). Similar changes in the electrical responses of *Limulus* photoreceptors have been observed after the pressure injection of large volumes of other substances; e.g., injections of 2×10^{-10} liters or more of mannitol solutions (Lisman and Brown).² Such volumes would be at least 25–80% of a typical cell volume.

We found that it was possible to use the changes in the receptor potential to optimize the volume of aequorin solution injected. During the injection of the aequorin solution, brief, dim flashes were applied repetitively to test the responsiveness of the cell; the aequorin solution was injected until the voltage responses to test flashes began to diminish. If the injection was stopped at this point, large aequorin responses could be measured and also the cell rapidly recovered its electrical responsiveness to stimuli. After aequorin injections of optimal size, the aequorin responses and concurrent receptor potentials elicited by successive stimuli of fixed size both increased in amplitude for a short time after the injection (Fig. 2) and then became relatively stable. Thereafter, the aequorin responses elicited by a series of uniform stimuli decreased slowly (Fig. 1 B), probably as a result of depletion of unreacted aequorin in the cell. The peak amplitude of the aequorin responses always declined with a half-time greater than 45 min, and large aequorin responses often could be measured 3–4 h after the injection.

After aequorin injections of optimal size, we could not detect any luminescence from unstimulated photoreceptors (Fig. 1 A); that is, there was no measurable difference in photomultiplier current before and after the injection as long as the cell was not stimulated. Any resting luminescence was so dim that it could not be detected against the background of the low ambient light levels present in our experiments.

We wish to emphasize that, when measured a few minutes after injections of optimal volume, the electrophysiological responses of the photoreceptor were apparently normal. Specifically, the sensitivity of the cell to brief light stimuli was reduced only slightly and recovered within a few minutes after the injection, the resting membrane voltage was not altered significantly, and the amplitude of the transient phase of the electrical response of the cell to prolonged illumination was large with respect to the plateau. The injections used in the present study did not produce lasting changes in the normal electrophysiological responses of the cells, except where specifically noted.

Estimation of the Absolute Volume Injected

In five cells the injection volume was measured as follows. Carrier-free $\text{H}_2^{35}\text{SO}_4$ was passed through a column of Chelex 100 (in the potassium form) to remove calcium contamination and then was evaporated to dryness. The labeled potassium sulfate was dissolved in an aequorin solution (1.4×10^{-4} M

aequorin). This solution, containing approximately 200 μC of $^{35}\text{SO}_4$ per microliter, was injected into *Limulus* ventral photoreceptors. Within 10 min after each injection, typical aequorin responses were measured and the responsiveness of the cell was normal. We judged these injections to be optimal. The preparation was digested in Pronase and the resulting solution was counted (in Aquasol) in a scintillation counter. Dilutions of the injection solution were also counted under the same conditions and the volume injected was calculated on the assumption that none of the injected radioactive sulfate had left the photoreceptor cell before it was digested. The mean volume injected (in five cells) was 2.5×10^{-11} liters. From the measurements of Clark et al. (1969) we estimate the volume of typical photoreceptor cells to be between 2.5 and 7.5×10^{-10} liters. Thus the volumes injected were about 3–10% of a typical cell volume, and the final aequorin concentration in the cells was of the order of 10^{-5} M.

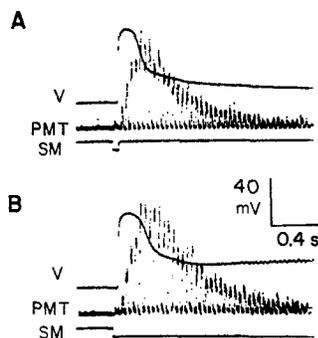


FIGURE 3

FIGURE 3. Influence of stimulus duration on aequorin responses and receptor potentials measured simultaneously. The PMT input aperture and the stimulus beam were chopped out of phase, allowing observation of the rising edge of the aequorin responses and luminescence changes during a prolonged stimulus. (A) Brief stimulus. (B) Prolonged stimulus at same intensity as in A. V: membrane voltage. PMT: photomultiplier current, arbitrary scale. SM: stimulus monitor. Stimulus irradiance: 3.15×10^{-6} W/cm².

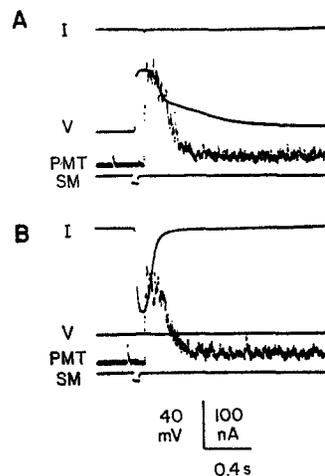


FIGURE 4

FIGURE 4. The effect of stabilizing membrane voltage on the aequorin response. (A) Aequorin response measured with the photoreceptor cell not voltage clamped. The artifact on the I trace indicates the time at which the shutter blocking the PMT was opened; the rising edge of the aequorin response is not recorded. (B) Aequorin response measured with the cell voltage clamped to resting voltage. Stimulus intensity same as in A. I: total clamp current. V: membrane voltage. PMT: photomultiplier current, arbitrary units. SM: stimulus monitor.

Time-Course of the Aequorin Response

The aequorin reaction *in vitro* responds to a step increase in calcium concentration with a half-time of less than 6 ms at 22°C (Hastings et al., 1969). The aequorin response recorded from *Limulus* photoreceptors (Fig. 3) rises with a half-time longer than 35 ms. Thus, the Ca-aequorin reaction itself does not significantly slow the rate of rise of the aequorin luminescence recorded from a photoreceptor; the aequorin response indicates that Ca_i rises slowly, and reaches a peak delayed with respect to the peak of the receptor potential (Fig. 3) or light-induced membrane current (Fig. 4).

When a *Limulus* ventral photoreceptor was stimulated with a brief light flash, the half-time of decay of the aequorin response was as short as 250–300 ms (Figs. 3 A, 4). The decay time increased if the cell had been very dark adapted or if the cell was bathed in seawater containing a greater than normal concentration of calcium. The half-time of decay of the aequorin response was not changed appreciably when a longer stimulus of the same intensity was applied (Fig. 3 B). In *Limulus* photoreceptors that produce normal electrical responses to light we have been unable to measure a sustained aequorin luminescence during a prolonged stimulus, even with very bright ($>2 \times 10^{-3}$ W/cm²) stimuli.

However, after the injection of a very large volume of aequorin solution into a *Limulus* ventral photoreceptor cell, it was possible to measure sustained aequorin luminescence during prolonged stimuli. After such an injection, the responsiveness of the cell to light was reduced and either no transient component of the receptor potential could be recorded (Fig. 5, 13 min) or the ratio of the amplitudes of the transient phase and the plateau phase was small (approaching unity) (Fig. 5, 54 min). In both of these conditions, sustained aequorin responses could be measured. As the cell recovered its responsiveness to light and as the ratio of the amplitudes of the transient and plateau phases increased, the peak of the transient aequorin response became larger and the sustained aequorin response became smaller (Fig. 5, 62 and 181 min).

Dependence of Aequorin Response on Membrane Voltage

The aequorin response elicited by a stimulus flash from a *Limulus* (or a *Balanus*) photoreceptor did not depend on having a light-induced change in membrane voltage. Stimuli of given size evoked similar aequorin responses whether membrane voltage was permitted to change or the cell was voltage clamped at resting voltage (Fig. 4). The relationship between membrane voltage and the peak amplitude of the aequorin response was also determined with the voltage clamp. The aequorin responses elicited by identical stimuli diminished in amplitude as the membrane potential was clamped at progressively more positive levels (Fig. 6). At the reversal potential for the light-induced

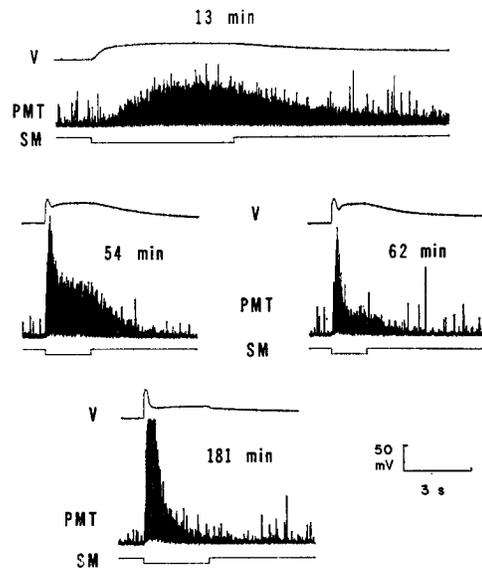


FIGURE 5. Aequorin responses from a *Limulus* ventral photoreceptor measured at various times after injection of a large volume of aequorin solution. Time after the injection identifies each set of traces. In each set of traces the upper trace is membrane voltage (V), the middle trace is photomultiplier tube current (PMT) in arbitrary units, and the lower trace is a stimulus monitor (SM). After the injection, the ratio of amplitude of peak transient phase to plateau increases with time for both the receptor potential and the aequorin response.

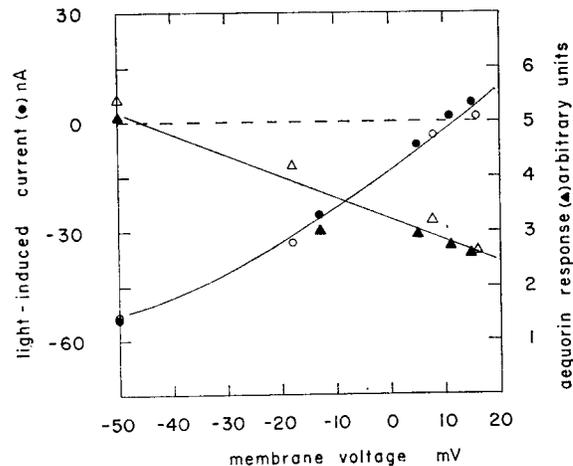


FIGURE 6. Influence of membrane voltage on aequorin response and light-induced current. Membrane voltage was maintained by a voltage clamp. Peak light-induced currents (circles) and corresponding peak aequorin responses (triangles) are plotted vs. membrane voltage. Hollow and filled symbols indicate measurements made during two successive experiments on the same cell.

current, the aequorin response was still large (about half of its value at resting voltage, Fig. 6). Aequorin responses can be elicited at membrane voltages as high as +50 mV. We have not attempted to clamp the membrane voltage to levels more positive than about +50 mV, since the properties of the cell undergo long-term changes after such large displacements of membrane voltage.

Influence of Stimulus Intensity and Adaptational State

In the photoreceptors of *Limulus* (and *Balanus*), the amplitude of the aequorin response is graded with stimulus intensity (Fig. 7 A,B). With our apparatus, stimuli having irradiances greater than 10^{-7} W/cm² are needed to elicit an aequorin luminescence large enough to be detected with a single stimulus. We have not attempted to average responses to dimmer stimuli. Brighter stimuli elicit larger, more prolonged, aequorin responses (Fig. 7 A).

The size of the aequorin response also depends on the adaptational state

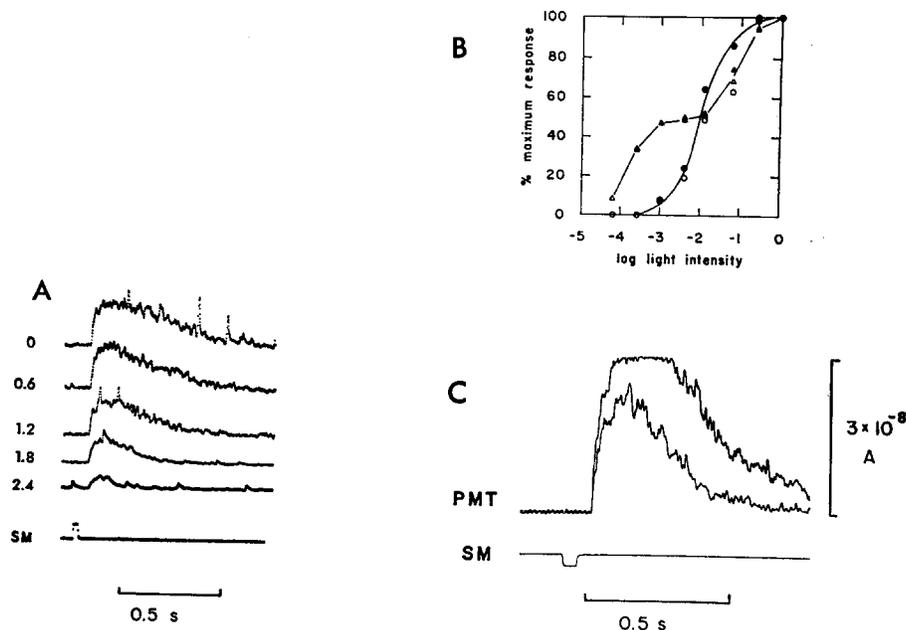


FIGURE 7. (A) Aequorin responses at various stimulus intensities. Log stimulus attenuation is marked at the left of each response. SM: stimulus monitor. Unattenuated stimulus: 4×10^{-4} W/cm² (B) Data from experiment illustrated in A. Closed and open symbols indicate separate runs. Circles: peak aequorin responses. Triangles: peak light-induced current. (C) Two aequorin responses elicited by identical stimuli. The larger response, in which the photometer amplifier saturated (full scale = 3×10^{-8} A), was preceded by 15 min in the dark. The smaller response was elicited 3 min after the first response. SM: stimulus monitor. PMT: photomultiplier tube current. In both A and C, the shutter blocking the PMT was opened after the stimulus flash.

of the cell. Fixed size stimuli elicit larger, more prolonged aequorin responses when the cell is dark adapted than when it is not (Fig. 7 C). Also, the amplitude of the aequorin response elicited by the second of a pair of equal stimuli depends strongly on the interstimulus interval. With pairs of equally bright stimuli, there is no aequorin response to the second flash for short interstimulus intervals; the size of the second response increases as the interstimulus interval is increased.

Influence of Intracellularly Injected EGTA

In previous work the intracellular injection of the calcium sequestering agent EGTA led to stabilization of the sensitivity of *Limulus* ventral photoreceptors (Lisman and Brown).² Fig. 8 shows the result of the iontophoretic injection of progressively increasing amounts of EGTA into a *Limulus* photoreceptor that had been previously injected with aequorin. As more EGTA was injected, the voltage responses to prolonged stimuli of constant intensity tended to have plateau values closer to the peak of the transient (more nearly square responses). Concurrently, the aequorin responses diminished (Fig. 8). When the receptor potential had become very square, the aequorin response was abolished (Fig. 8 D). The intracellular aequorin had not lost its activity during this time; at the end of the experiment, the cell was intentionally torn with the micropipette and aequorin luminescence was observed.

Influence of Ca_o and Mg_o

It is known that changes in Ca_o influence the amplitudes of the electrical responses to light in both *Limulus* (Millecchia and Mauro, 1969 *a,b*; Lisman and Brown, 1972) and *Balanus* (Brown et al., 1970) photoreceptors. However, in *Limulus* photoreceptors bathed in sodium-free solutions, there is no noticeable change in the reversal voltage of the light-induced current when Ca_o is changed (Brown and Mote, 1974); that is, a light-induced calcium current cannot be detected with voltage-clamp techniques. In *Balanus* photoreceptors, in the absence of extracellular sodium ions, the reversal voltage changes approximately 20 mV for a 10-fold change of Ca_o ; thus, for barnacle photoreceptors there is voltage-clamp evidence for a light-induced calcium current (Brown et al., 1970). To study this difference in the ionic currents in the two species, we changed Ca_o while observing changes in intracellular aequorin luminescence.

In *Limulus* ventral photoreceptors, when extracellular calcium is increased the electrical responses to light stimuli of fixed size become smaller (Lisman and Brown, 1972), but the aequorin responses become larger (Fig. 9 C, D). When Ca_o is reduced, the electrical responses elicited by stimuli of fixed size become larger (Millecchia and Mauro, 1969 *a,b*; Lisman and Brown, 1972; Brown and Mote, 1974), but the aequorin responses become smaller (Fig.

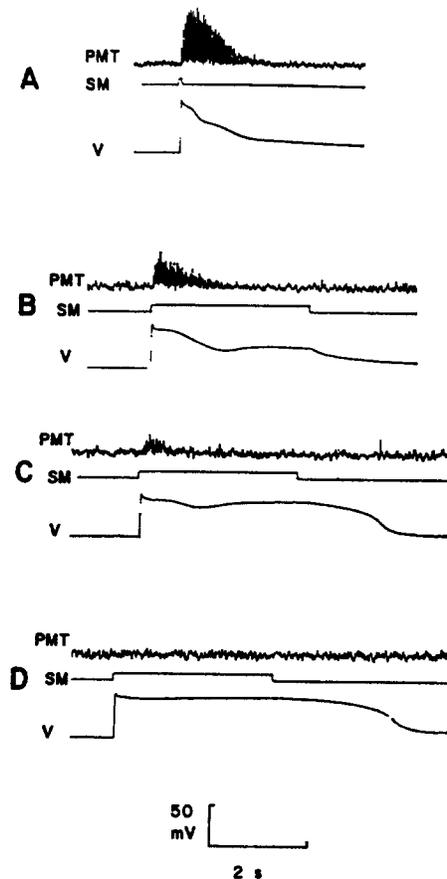


FIGURE 8. Effect of progressive EGTA injection into a *Limulus* photoreceptor previously injected with aequorin. (A) Response to a brief stimulus before the cell was impaled with the EGTA-filled electrode. (B, C, D) After impalement with EGTA-filled electrode. EGTA was iontophoretically injected between stimuli. Notice that as the receptor potential becomes more square the aequorin response diminishes. PMT: photomultiplier tube current (arbitrary scale). SM: stimulus monitor. V: membrane voltage.

9 A,C). If a *Limulus* ventral photoreceptor cell is bathed in a solution in which all the calcium and magnesium are removed and 5 mM EGTA is added, the membrane voltage becomes more positive. Within 1–2 min, membrane voltage approaches (and sometimes becomes more positive than) reversal voltage for the electrical response to light (Fig. 10). With the cell in this condition, there is still a large light-induced change in membrane conductance as measured with a voltage clamp (J. Brown, unpublished observation); when elicited by a prolonged stimulus this conductance change has both transient and plateau phases. Also, the aequorin response can be large

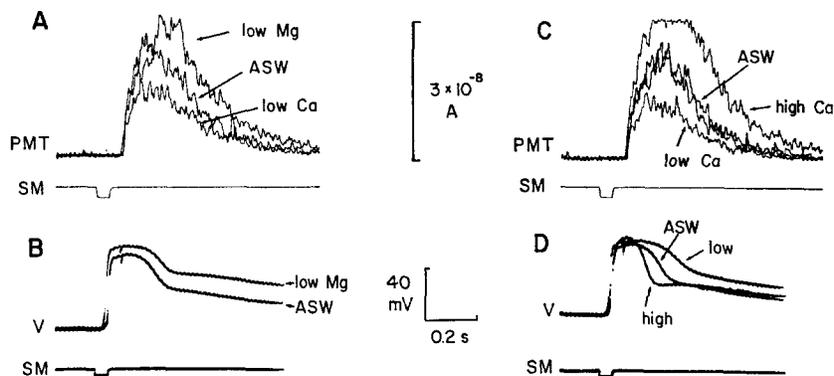


FIGURE 9. Influence of Ca_o and Mg_o on aequorin responses and receptor potentials. The traces in B were recorded simultaneously with the corresponding traces in A; those in D were recorded simultaneously with the corresponding traces in C. The receptor potential in low Ca-SW is not shown in B; a similar record is shown in D. The photometer amplifier saturated in the high-Ca trace in C at 3×10^{-8} A. V: membrane voltage. PMT: photomultiplier tube current. SM: stimulus monitor. The transient artifact in the voltage records near the peak of the receptor potential occurred when the electromechanical shutter blocking the PMT was energized.

and prolonged (Fig. 10). In four *Limulus* cells tested with no calcium, no magnesium, and 5 mM EGTA in the bath for 4–8 min, the aequorin response became no smaller than 30% of that measured in ASW.

In *Balanus* photoreceptors, no aequorin responses were detectable when extracellular calcium was replaced isosmotically by sucrose. These observations support the previous conclusion that light induces an inward calcium current in *Balanus* photoreceptors.

Since calcium and magnesium ions both influence the aequorin reaction itself, we also studied the effects of changing Mg_o on the aequorin response. In *Limulus* ventral photoreceptors, when extracellular magnesium ions were replaced by either Tris ions or sucrose, the electrical responses elicited by light stimuli of fixed size became larger (Brown and Mote, 1974); the aequorin responses also became larger (Fig. 9 A,B). Thus, changes in Mg_o influenced the electrical responses to light and the aequorin responses in the same direction, although changes in Ca_o influenced them in opposite directions. In *Balanus* photoreceptors, we observed no effect on either the receptor potential or the aequorin response when Mg_o was replaced by sucrose.

Balanus Photoreceptors

The aequorin responses recorded from *Balanus* photoreceptors were similar to those recorded from *Limulus* photoreceptors, with two notable exceptions. First, as described above, the aequorin responses from *Balanus* photoreceptors

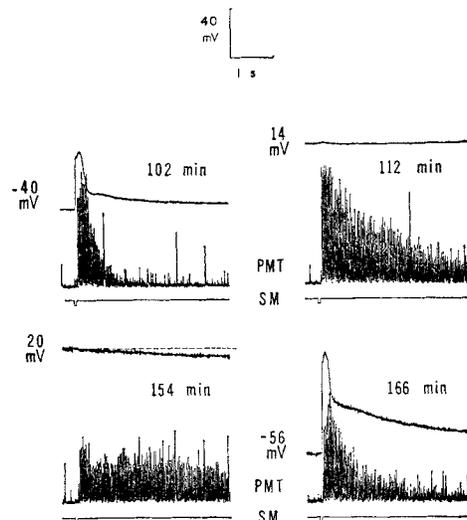


FIGURE 10. Aequorin responses measured with the cell bathed in 0 Ca, 0 Mg, 5 mM EGTA. Time after aequorin injection indicated on each set of traces. In each set of traces: upper trace, membrane voltage recorded via a platinum wire in the injection pipette; PMT: photomultiplier tube current; SM: stimulus monitor. Numbers to the left of each voltage trace indicate membrane voltage relative to reversal voltage for the receptor potential, which was arbitrarily taken at +15 mV. Traces at 102 and 166 min in ASW. Traces at 112 and 154 min were recorded 3 min after perfusion with seawater containing 0 Ca, 0 Mg, 5 mM EGTA had begun. The aequorin responses recorded in 0 Ca, 0 Mg, 5 mM EGTA from this cell were the largest of those from the four cells tested. The cell was allowed to recover in ASW between 112 min and 151 min.

became undetectable when extracellular calcium was removed, whereas large aequorin responses could be measured from *Limulus* photoreceptors bathed in the same solution. Second, although in the photoreceptors of *Balanus* the aequorin responses elicited by brief stimuli had decay times similar to those measured from *Limulus* photoreceptors (Fig. 11 A), with prolonged stimuli the rate of decay of aequorin luminescence became much slower (Fig. 11 B). Furthermore, a sustained aequorin luminescence could be measured during a prolonged, bright stimulus (Fig. 11 B, C, D). The sustained aequorin luminescence decayed with a half-time of about 300 ms when the stimulating light was turned off. With the apparatus presently available to us we were unable to observe a sustained aequorin luminescence during the plateau phase of the receptor potential recorded from *Limulus* photoreceptors injected with optimal volumes of aequorin solution. Responses similar to those normally recorded from *Balanus* could be elicited from *Limulus* cells only after the injection of very large volumes of aequorin solutions (see Fig. 5).

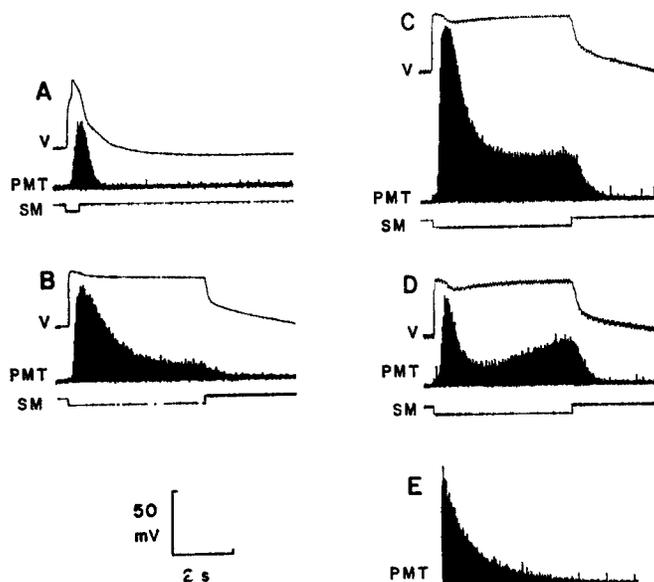


FIGURE 11. Aequorin responses and receptor potentials measured simultaneously from *Balanus* photoreceptors. Responses to (A) a brief stimulus and (B) a prolonged stimulus. (C, D) Another cell which was dark adapted for 30 min before (C) and 3 min before (D). (E) Decay of aequorin luminescence from aequorin injected into ASW. V: membrane voltage. PMT: photomultiplier tube current. SM: stimulus monitor.

DISCUSSION

Do Changes of Ca_i Produce the Aequorin Response?

The luminescent response of aequorin injected into *Limulus* and *Balanus* photoreceptor cells demonstrates either (a) that as a result of illumination the concentration of a substance which activates the luminescent reaction inside these cells rises, or (b) that an activator of the reaction is constantly present, and that illumination leads to a fall of the intracellular concentration of some substance which inhibits the aequorin reaction. Of those ions known to activate the luminescent reaction of aequorin (Izutsu et al., 1972; Shimomura and Johnson, 1973), the only one likely to be found in sufficient concentration in photoreceptors is calcium. Two substances present in living cells are known to inhibit the aequorin reaction magnesium and hydrogen ions. Magnesium apparently acts as a simple competitive antagonist to calcium, and has an appreciable effect in concentrations as low as 1 mM (Blinks, 1973). We have no direct measure of either the magnitude of, or changes of, intracellular free magnesium concentration in these invertebrate photoreceptors. If Mg_i is similar to that of cells in which it has been estimated ($Mg_i \approx 1-5$ mM; Baker and Crawford, 1972; Veloso et al., 1973), it is

sufficient to inhibit significantly the Ca-aequorin reaction. However, for a transient fall in Mg_i to be responsible for the aequorin response, there would have to be some hitherto unsuspected mechanism for the light-induced sequestration of magnesium ions. It is unlikely that a change in Mg_i could arise from a light-induced magnesium flux across the cell membrane; no significant fraction of the light-induced current is carried by magnesium ions, since removal of extracellular magnesium leads to no measurable change in reversal voltage of the response to light (Brown and Mote, 1973). Hence, we think it unlikely that there are light-induced changes in Mg_i large enough to account for a large change of the inhibition of the aequorin reaction.

Although hydrogen ions also inhibit the aequorin reaction, changes in pH do not appreciably affect the reaction within 1 pH unit on either side of neutrality (Shimomura and Johnson, 1973). Therefore, we ought to consider whether changes in pH_i are likely to exceed that range during the response to illumination. A light-induced change in pH_i might arise either (a) from a transmembrane flux of hydrogen ions or (b) from changes of intracellular binding sites for protons, such as those on rhodopsin, which become available after isomerization of the rhodopsin chromophore.

First, if the membrane became completely permeable to hydrogen ions during illumination, then equilibrium voltage for hydrogen ions would approach membrane voltage. At the peak of the receptor potential, $E_H \simeq V_m = +20$ mV, pH_o is buffered at 7.8, and $pH_i \leq 8.1$. Assuming pH_i is near neutrality in resting cells, a permeability change to hydrogen ions could not itself perturb pH_i enough to cause a large change in the aequorin reaction.

Second, the proton binding sites uncovered during the dark reactions of invertebrate rhodopsins have pK values which vary widely among species (pK = 9.1 in *Todarodes*, Hara and Hara, 1972; pK = 6.8 in *Eledone*, Hamdorf et al., 1968). To our knowledge, the properties of the proton binding sites have not been reported for either *Limulus* or *Balanus* rhodopsin; therefore, we cannot say whether pH_i would rise or fall as a result of the rhodopsin chemistry. However, we can estimate the maximum change in pH_i which might arise in a *Limulus* ventral photoreceptor cell as follows. If we assume (a) that there is no intracellular pH buffering, (b) that each rhodopsin molecule which absorbs a photon reveals one proton binding site, and (c) that about 0.5% of the incident photons are effectively absorbed (Murray, 1966; Millecchia and Mauro, 1969 a), then knowing the irradiance of a typical stimulus (3.75×10^{-6} W/cm², Fig. 4), we calculate that the change of intracellular hydrogen ion concentration during 0.2 s of illumination is about 10^{-7} M. Therefore, if the intracellular pH is close to neutrality in a resting cell, the change of pH_i induced by a 0.2-s stimulus would be less than 0.5 pH unit, even if the inside of the cell were unbuffered. However,

the inside of the cell undoubtedly is buffered against changes in pH_i , and stimuli of much shorter duration (0.05 s in Fig. 4 A) can elicit aequorin responses. Hence, it appears unlikely that the rhodopsin chemistry can be responsible for large enough perturbations of pH_i to have an appreciable effect on the Ca-aequorin reaction.

In addition, we have done a preliminary experiment in which a pH buffer solution (morpholinopropane sulfonic acid, "MOPS") containing radioactive sulfate, was injected into a *Limulus* photoreceptor previously injected with aequorin. After the injection of 1.1×10^{-10} liters of 1 M MOPS (adjusted to pH 7 with KOH), aequorin responses could still be observed. In this cell, the intracellular MOPS concentration must have been greater than 100 mM. This single observation makes it seem very improbable that changes in pH_i can account for the light-elicited changes of aequorin luminescence. Having no knowledge of other intracellular substances which might inhibit the aequorin reaction, we conclude that the light-induced increase of aequorin luminescence results from a light-elicited increase in free calcium concentration inside the photoreceptor cell.

In photoreceptor cells having normal sensitivity to light, intracellular aequorin is depleted very slowly in the dark or if stimuli are given infrequently. Because of this finding, we presume that the free Ca_i in a dark-adapted photoreceptor must be low, as has been found in other neurons (Baker, 1972). Since we cannot detect a resting luminescence from a normal dark-adapted cell with our apparatus and we do not know Mg_i or whether other inhibitors of the aequorin reaction are present, we cannot estimate the absolute Ca_i in the dark, or the absolute peak Ca_i attained.

Possible Mechanisms for the Rise in Ca_i

At least three mechanisms might be involved in the production of the light-induced rise in Ca_i .

First, light could elicit a change in membrane permeability to calcium; that is, calcium could carry a fraction of the inward current induced by illumination. Consistent with this idea is the result (in both *Limulus* and *Balanus* photoreceptors) that changing Ca_o changes the size of the aequorin response in the direction predicted for a light-induced increase in membrane permeability to calcium. Furthermore, as membrane voltage is made more positive, the aequorin response becomes smaller. This also agrees with there being a light-induced change in calcium permeability, since the equilibrium potential for calcium ought to be a large positive voltage (e.g., if $\text{Ca}_i \simeq 10^{-7}$ M then $E_{\text{Ca}} \simeq +150$ mV).

Second, calcium may be released from intracellular stores. In *Limulus* photoreceptors, the entire aequorin response cannot be ascribed to an influx of calcium across the cell membrane because an aequorin response can be

recorded with 5 mM EGTA added to and calcium removed from the bathing solution. Thus, light may release calcium from an intracellular (or membrane) store in addition to (or instead of) permitting it to enter the cell by a change in membrane permeability. This could be analogous to the situation in some types of muscle, where stimulation both causes calcium to enter the sarcoplasm through the sarcolemma and causes the release of calcium from the sarcoplasmic reticulum (Ebashi and Endo, 1968; Hagiwara et al., 1968; Reuter, 1972). Further, since the amount of calcium in the releasable store inside the photoreceptor might depend on Ca_o (as it does in mammalian cardiac muscle, Beeler and Reuter, 1970), the influence of Ca_o on the aequorin response does not unequivocally demonstrate the existence of a light-induced inflow of calcium ions across the cell membrane.

Third, calcium ions might enter the cell as a result of a Na-Ca exchange mechanism (Baker, 1970). Light induces a net influx of sodium ions, and there is indirect evidence for a Na-Ca exchange mechanism in *Limulus* ventral photoreceptors (Lisman and Brown, 1972; Brown and Lisman, 1972). Both diminishing Ca_o and making the membrane voltage more positive should reduce the influx of calcium ions through a Na-Ca exchange mechanism. Even if the Na-Ca exchange did not occur rapidly enough to be directly responsible for the aequorin response, it might participate in the regulation of releasable intracellular stores of calcium.

Implications of the Waveform of the Aequorin Response

In experiments performed with a rapid-mixing stopped-flow photometer, Hastings et al. (1969) found that aequorin luminescence followed step changes in calcium concentration with a half-time of less than 6 ms at 22°C. Since this rate is at least five times that of the fastest aequorin response observed in our experiments, the kinetics of the aequorin-calcium reaction are not likely to have slowed significantly the rate of rise of luminescent intensity. Thus, the finding that the peak of the aequorin response is delayed with respect to the peak of the electrical response to light indicates that the maximum value of Ca_i is reached after the peak of the transient phase of the electrical response to light.³

The decline of luminescent intensity during or at the end of a stimulus might result from (a) a rise in the intracellular concentration of an inhibitor of the aequorin reaction, (b) depletion of active aequorin, or (c) a decline in

³ Although the stoichiometry of the light-producing reaction of aequorin with calcium is not clear (Blinks, 1973), it has been found in some in vitro experiments (Ashley, 1970; Baker et al., 1971) that the intensity of luminescence is proportional to the calcium ion concentration raised to a power greater than one. If this is also true within the photoreceptor cell, the half-time for the rise of luminescent response will be longer than that for the rise of Ca_i . Nonetheless (except for the small delay due to the Ca-aequorin reaction time), the peak of the luminescent response will occur at the same time as the peak of the calcium transient unless saturation of the aequorin reaction occurs.

Ca_i . First, we have already indicated that there is no independent evidence in favor of there being a delayed change in either Mg_i or pH_i large enough to account for the declining phase of the aequorin response. Second, in these experiments a negligible proportion of the injected aequorin could have been depleted in a single response, since many responses could be elicited after a single injection without marked diminution in response. It might be argued that during each aequorin response the aequorin becomes exhausted in some intracellular compartment which must be refilled by diffusion before another aequorin response can occur. This is improbable because even in an excess of calcium ions the consumption of aequorin is limited by a step in the reaction sequence to a half-time of about 500 ms at 22°C (calculated from Hastings et al., 1969; see also Fig. 11 E). The luminescence can be extinguished more rapidly by the sequestration of calcium ions (Hastings et al., 1969). Since the aequorin luminescence which we measure from invertebrate photoreceptors often decays with a half-time substantially shorter than 400 ms, free calcium ions must be removed from the intracellular space containing the active aequorin molecules. This removal of calcium might be due either to an active, energy-dependent process or to the binding of calcium to sites accessible during and after the response of the photoreceptor to light. Furthermore, under certain conditions the aequorin responses of both *Balanus* and *Limulus* photoreceptors are maintained during prolonged stimuli, showing directly that the aequorin has not been depleted within the appropriate compartment. We conclude that during a prolonged light stimulus, the Ca_i first rises, then falls to a new level (which for normal *Limulus* photoreceptors is undetectable by our apparatus).

Previous work on *Limulus* ventral photoreceptors indicated that intracellular calcium ions play an important role in the process of light adaptation. The injection of EGTA into a cell previously injected with aequorin led to a concurrent reduction of the aequorin response and of the decline of the receptor potential from transient phase to plateau. When the aequorin response was eliminated, the receptor potential became very square (see also Lisman and Brown).² These results suggest that the light-induced rise of Ca_i which tends to be prevented by intracellular EGTA is responsible for both the aequorin response and the decline from transient phase to plateau of the receptor potential in the normal photoreceptor. Furthermore, we have found one condition in which we have been able to measure a resting aequorin luminescence (i.e., shortly after the intracellular injection of a large volume of aequorin solution). In this condition the Ca_i most likely was elevated, and the sensitivity of the cell to light was depressed. When the sensitivity returned to normal, no resting luminescence was detected and the Ca_i presumably had become lower. This result indicates that a rise in Ca_i correlates

with a decrease in the sensitivity of a dark-adapted *Limulus* ventral photoreceptor.

We have argued that the aequorin response reflects an increase of Ca_i and that the kinetics of the aequorin reaction are fast enough to follow changes of Ca_i . If this is so, then the Ca_i rises from a low value in the dark to a peak which occurs during the decline of the electrical response from transient to plateau. This is the interval during which the sensitivity declines (Lisman and Brown).¹ If the stimulus is prolonged, the sensitivity reaches a steady minimum during the plateau phase of the electrical response, whereas the Ca_i falls to some new low level. When the stimulus is removed, the sensitivity increases (dark adaptation) and the Ca_i returns to a still lower value in the dark. The value of the Ca_i cannot then be the direct signal of the level of light adaptation. Although Ca_i rises as the cell light adapts, the Ca_i then falls during illumination whereas the cell remains steadily light adapted. That is, the value of Ca_i is not at all times monotonically related to the value of the sensitivity.

We tentatively suggest that the control of sensitivity in a *Limulus* ventral photoreceptor depends on calcium being reversibly bound to appropriate sites of high affinity. Hence, during the peak of the aequorin response, the large increase of Ca_i might lead to a large number of occupied binding sites (and a concomitant reduction of sensitivity). During a prolonged stimulus, the binding reaction might reach a steady state in which a lower Ca_i would keep a high concentration of binding sites occupied by Ca (and hence keep the sensitivity steadily reduced). That is, the binding sites would behave like a "leaky" integrator for changes of Ca_i . The relation between Ca_i and sensitivity which we have proposed is similar to that proposed by Ashley and Moiescu (1972) to account for the relationship between Ca_i and tension in barnacle muscle. In summary, the direct detection of changes in intracellular free calcium concentration in the present study supports the hypothesis that an increase in Ca_i is a step in the sequence of events leading to light adaptation in *Limulus* ventral photoreceptors.

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