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CALCIUM ION, AN INTRACELLULAR MESSENGER OF LIGHT ADAPTATION, ALSO PARTICIPATES IN EXCITATION OF *LIMULUS* PHOTORECEPTORS

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SUMMARY

1. Photoreceptor cells of *Limulus* ventral eyes were bathed in artificial sea water (ASW) that contained 10 mM-EGTA and no added Ca^{2+} (EGTA–ASW). Test flashes elicited responses that increased to a maximum size within 10 min in EGTA–ASW but did not change further when dark-adapted cells were bathed for an additional 35 min in this solution.

2. Light responses progressively declined from this maximum size if the cells were repetitively illuminated in EGTA–ASW. In this state of reduced responsiveness, response amplitudes were further reduced by intracellular ionophoretic injection of EGTA; response amplitudes were increased by intracellular ionophoretic injection of Ca^{2+}. Both of these findings are opposite to what is normally observed for cells bathed in ASW. Also, after repetitive illumination in EGTA–ASW, both the slope of the response *versus* intensity relationship became steeper and light responses often had a delayed increase in amplitude.

3. The light responses and the response *versus* intensity relation returned to normal when the bathing medium was changed back to ASW containing 10 mM-Ca^{2+}.

4. The light-induced rise in luminescence recorded from photoreceptors injected with the photoprotein aequorin (the 'aequorin response') declined by at most 50% after dark-adapted photoreceptors were bathed in EGTA–ASW for 45 min. However, the aequorin response progressively declined by 98% if cells were repetitively illuminated while bathed in EGTA–ASW.

5. The total intracellular Ca content of whole end-organs was measured by atomic absorption spectroscopy. Total intracellular Ca content did not change significantly while photoreceptors were bathed in EGTA–ASW even after repetitive illumination.

6. We suggest that cytosolic Ca^{2+} is required by one or more steps in the mechanisms that link rhodopsin isomerization to both (i) an increase in the conductance of the cell membrane to Na^+ and (ii) a release of Ca^{2+} from a light-labile store.

INTRODUCTION

Lisman & Brown (1972) proposed that Ca^{2+} is an intracellular messenger for light adaptation in *Limulus* ventral photoreceptor cells. This hypothesis is supported by

several lines of evidence. Illumination causes an increase in the concentration of Ca^{2+} in the cytosol, $[Ca^{2+}]_i$. When cells are bathed in Ca^{2+}-free sea water, illumination initially evokes a similar increase in $[Ca^{2+}]_i$, indicating that Ca^{2+} is released from an intracellular, light-labile store (Brown & Blinks, 1974; Brown, Brown & Pinto, 1977; Maaz & Stieve, 1980). During continued illumination in Ca^{2+}-free sea water, the light-induced increase in $[Ca^{2+}]_i$ becomes small or disappears (Maaz & Stieve, 1980). The light-induced increase in $[Ca^{2+}]_i$ is localized (Harary & Brown, 1984) probably to the rhabdomeric lobe of the photoreceptor. The sensitivity of photoreceptors to light is reduced when $[Ca^{2+}]_i$ is increased by pressure injection of Ca^{2+}–EGTA solutions (Lisman & Brown, 1975*b*) or by ionophoretic injection of Ca^{2+} (Lisman & Brown, 1972; Fein & Charlton, 1977). The site of desensitization is localized to the site of Ca^{2+} injection (Fein & Lisman, 1975). Also, the light-induced changes in sensitivity are blocked by intracellular injection of Ca^{2+} buffers (Lisman & Brown, 1975*b*).

The present paper presents a result paradoxical to the hypothesis of Lisman & Brown (1972). Photoreceptor cells were bathed in Ca^{2+}-free sea water that contained EGTA and were repetitively illuminated; ionophoretic injection of Ca^{2+} into the cytosol of such cells led to an increase in the amplitude of the light response. Therefore, we suggest that $[Ca^{2+}]_i$, in addition to its role in light adaptation, is required by one or more steps in the excitation mechanism that links rhodopsin isomerization to the change in membrane conductance. Some of these results have been published in preliminary form (Bolsover & Brown, 1982).

METHODS

The ventral rudimentary eye of *Limulus polyphemus* was desheathed, pinned into a silicon rubber (Sylgard 184, Dow-Corning, Midland, MI) dish and bathed with Pronase (grade B, Calbiochem, La Jolla, CA; 20 mg ml^{-1}) in artificial sea water (ASW) for 1 min. Thereafter the nerve was bathed in ASW composed of 422 mM-NaCl, 10 mM-KCl, 22 mM-$MgCl_2$, 26 mM-$MgSO_4$, 10 mM-$CaCl_2$ and 10 mM-Tris Cl, pH 7·8, or in EGTA–ASW in which the 10 mM-$CaCl_2$ was replaced by 10 mM-Na_2 ethyleneglycol-bis-(β-aminoethylether)*N,N,N′,N′*-tetraacetate (Na_2 EGTA). The total Ca^{2+} in different batches of EGTA–ASW was determined to be in the range 15–50 μM by atomic absorption spectroscopy. To make Ca EGTA–ASW, 10 mM-$CaCl_2$ was replaced by 9·091 mM-$Ca(OH)_2$ and 10 mM-Na_2 EGTA.

In order to estimate the concentration of free Ca^{2+} and Mg^{2+} in EGTA–ASW and Ca EGTA–ASW, we calculated the apparent stability constants of Ca EGTA and Mg EGTA (Blinks, Wier, Hess & Prendergast, 1982) from the stability constants for EGTA given by Martell & Smith (1974). We corrected for the effect of ionic strength by the procedure of Harafuji & Ogawa (1980). In EGTA–ASW that contained 50×10^{-6} M total Ca, the free Ca^{2+} concentration was estimated by the Newton–Raphson iterative method (Bartfai, 1979) to be $0{\cdot}43 \times 10^{-9}$ M and free Mg^{2+} concentration was estimated to be 40×10^{-3} M. In Ca EGTA–ASW, free Ca^{2+} concentration was estimated to be $0{\cdot}98 \times 10^{-6}$ M and free Mg^{2+} concentration was estimated to be 47×10^{-3} M.

Electrophysiology

Experiments were performed at room temperature (22 ± 1 °C). Results are expressed as mean ± standard error of the mean.

Stimulus light from a tungsten iodide lamp was passed through an electromechanical shutter, an infra-red blocking filter (KG1, Schott, Duryea, PA) and neutral density filters before being focused on the preparation. 1 W cm^{-2} corresponded to approximately 2×10^{10} photons s^{-1} absorbed by the cell. Micropipettes filled with 2 M-KCl were used to record membrane voltage and to pass voltage-clamp current as described previously (Millecchia & Mauro, 1969*a*,*b*; Brown, Harary &

Waggoner, 1979). Ionophoretic injections were made into a voltage-clamped cell from a third intracellular micropipette. Injections of Ca^{2+} were made by passing positive current out of micropipettes filled with a solution that contained 90 mM-$Ca(OH)_2$, 100 mM-EGTA and 100 mM-Tris, pH 4·6. Control injections of K^+ were made by passing positive current out of micropipettes filled with a solution that contained 180 mM-KOH, 100 mM-EGTA and 100 mM-Tris, pH 4·7. Injections of EGTA were made by passing negative current out of micropipettes containing either the KOH–EGTA–Tris solution or 300 mM-K_2 EGTA at pH 8·0. The Ca^{2+}-sensitive photoprotein aequorin was injected into single cells as described previously (Brown & Blinks, 1974). Light emitted by intracellular aequorin was collected with a microscope objective (H32, NA 0·60, Leitz, Wetzlar, West Germany) and focused directly onto the photocathode of a photomultiplier tube (R928P, Hamamatsu, Hamamatsu City, Japan) operated at 1000 V. The photomultiplier tube was cooled to approximately −65 °C with a dry ice–methanol mixture. The photon frequency was measured using a photon counter (Ortec, Oak Ridge, TN).

Atomic absorption

Total Ca was measured in whole end-organs. From each pair of end-organs from a single animal, one control end-organ was bathed in darkness in ASW for 30 min then bathed for 5 min in darkness in Zn EGTA–ASW (422 mM-NaCl, 10 mM-KCl, 58 mM-$MgCl_2$ (Puratronic grade, Johnson Matthey, Royston, U.K.), 50 μM-$ZnCl_2$, 10 mM-EGTA and 10 mM-Tris, pH 7·8). Zn EGTA was included as an extracellular marker. The free Zn^{2+} in Zn EGTA–ASW was calculated as $0{\cdot}95 \times 10^{-11}$ M using the stability constant log $K_{Zn} = 12{\cdot}7$ ($I = 0{\cdot}1$) (Martell & Smith, 1974). This concentration of free Zn^{2+} should have no physiological effect. The second end-organ from each pair was treated in one of two ways: (1) the end-organ was bathed in EGTA–ASW for 30 min in darkness, or (2) the end-organ was bathed in EGTA–ASW for 30 min and illuminated using a xenon flash (Strobotac, General Radio, Concord, MA) once every 10 s (intensity at the preparation 2×10^{-5} J cm^{-2} flash^{-1}). After either treatment the end-organ was bathed for 5 min in Zn EGTA–ASW in darkness and the perfusion dish drained before the room lights were turned on. The end-organ together with adhering Zn EGTA–ASW was transferred using a polyethylene scoop to a pre-washed, 2 ml plastic test-tube containing 200 μl 0·1 N-HCl (Ultrex grade, J. T. Baker, Philipsburg, NJ) plus 11 μM-La_2O_3 (Ultrex grade) to displace Ca^{2+} from high-affinity binding sites. The samples were frozen, thawed, ultrasonicated for 1 min (G112SP1T bath ultrasonicator, Laboratory Supplies Co, Hicksville, NY) and centrifuged for 15 min at 15000 *g*. 100 μl supernatant was taken for measurements of Ca and Zn using an atomic absorption spectrophotometer (Perkin–Elmer 5000 with HGA-400 graphite furnace and argon gas flow). Blanks contained 200 μl HCl–La_2O_3 solution. The amount of extracellular Ca present in each sample was calculated from the measured Zn content; this value was subtracted from the total Ca in each sample to calculate intracellular Ca. The pellet was analysed for protein by the method of Schaffner & Weissman (1973). For each pair of end-organs from the same animal, Ca per unit protein in the end-organ bathed in EGTA–ASW was expressed as a percentage of Ca per unit protein in the end-organ bathed in ASW. By expressing the results in this way we eliminate any variation of intracellular Ca content that results from systematic differences between animals.

RESULTS

Cells bathed in EGTA–ASW

Repetitive illumination elicited receptor potentials whose amplitudes changed when the solution bathing ventral photoreceptor cells was changed from ASW to EGTA–ASW. The receptor potentials became larger during the first few minutes in EGTA–ASW. Longer periods of repetitive illumination in EGTA–ASW led to reduction in amplitude of the receptor potentials and to other changes that are described below.

Fig. 1*A* illustrates the time course of the changes in amplitude for receptor potentials in EGTA–ASW. Individual receptor potentials from the recording shown in Fig. 1*A* are displayed at a higher sweep speed in Fig. 1*B*. These records illustrate that both the peak and plateau of receptor potentials became larger, as was reported

by Millecchia & Mauro (1969*a*) for cells bathed in sea water that contained no added Ca salts. The factor by which the peak of the receptor potential initially increased was in the range 1·2–9. Some cells were impaled by two or three micropipettes and the sensitivity (defined as the peak light-induced current per unit irradiance) was measured by a voltage clamp (Brown *et al.* 1979). Sensitivities were in the range 0·001–0·07 A W^{-1} cm^2) for cells bathed in ASW; cells impaled with three micropipettes

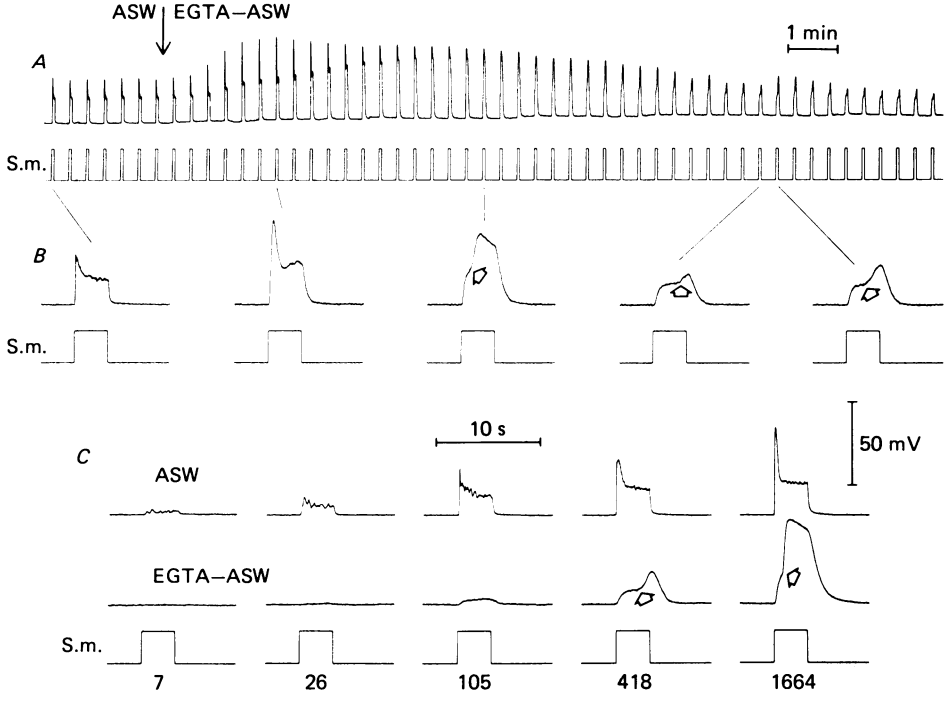

Fig. 1. Light responses in EGTA–ASW. A ventral photoreceptor cell was impaled with a single micropipette containing 2 M-KCl. *A*, receptor potentials elicited by repetitive flashes ($4·2 \times 10^{-7}$ W cm^{-2}, 3 s duration). At the arrow the solution bathing the preparation was changed from ASW to EGTA–ASW. S.m.: stimulus monitor. *B*, selected receptor potentials from *A* replotted at a faster sweep speed. Open arrows in *B* and *C* indicate the onset of a delayed increase in receptor potential amplitude. *C*, each horizontal row shows receptor potentials elicited by a series of flashes of increasing intensity. Each series was begun after the cell was in darkness for 5 min. Top row: in ASW, before the record in *A* began. Bottom row: in EGTA–ASW, 5 min after the end of the record in *A*. The light intensity in nW cm^{-2} is indicated below the stimulus monitor.

had the lowest sensitivity. The sensitivity increased by a factor in the range 6–60 within 10 min after the bath was changed to EGTA–ASW. The largest increases in sensitivity were seen in those cells that had the lowest sensitivity when bathed in ASW; $[Ca^{2+}]_i$ may have been artifactually high in those cells. However, changing the bath to EGTA–ASW led to an increase in the responsiveness of all cells, including those impaled by a single micropipette in which dim illumination elicited large discrete events (Millecchia & Mauro, 1969*a*). Therefore we do not ascribe all the increased responsiveness to an artifact of impalement.

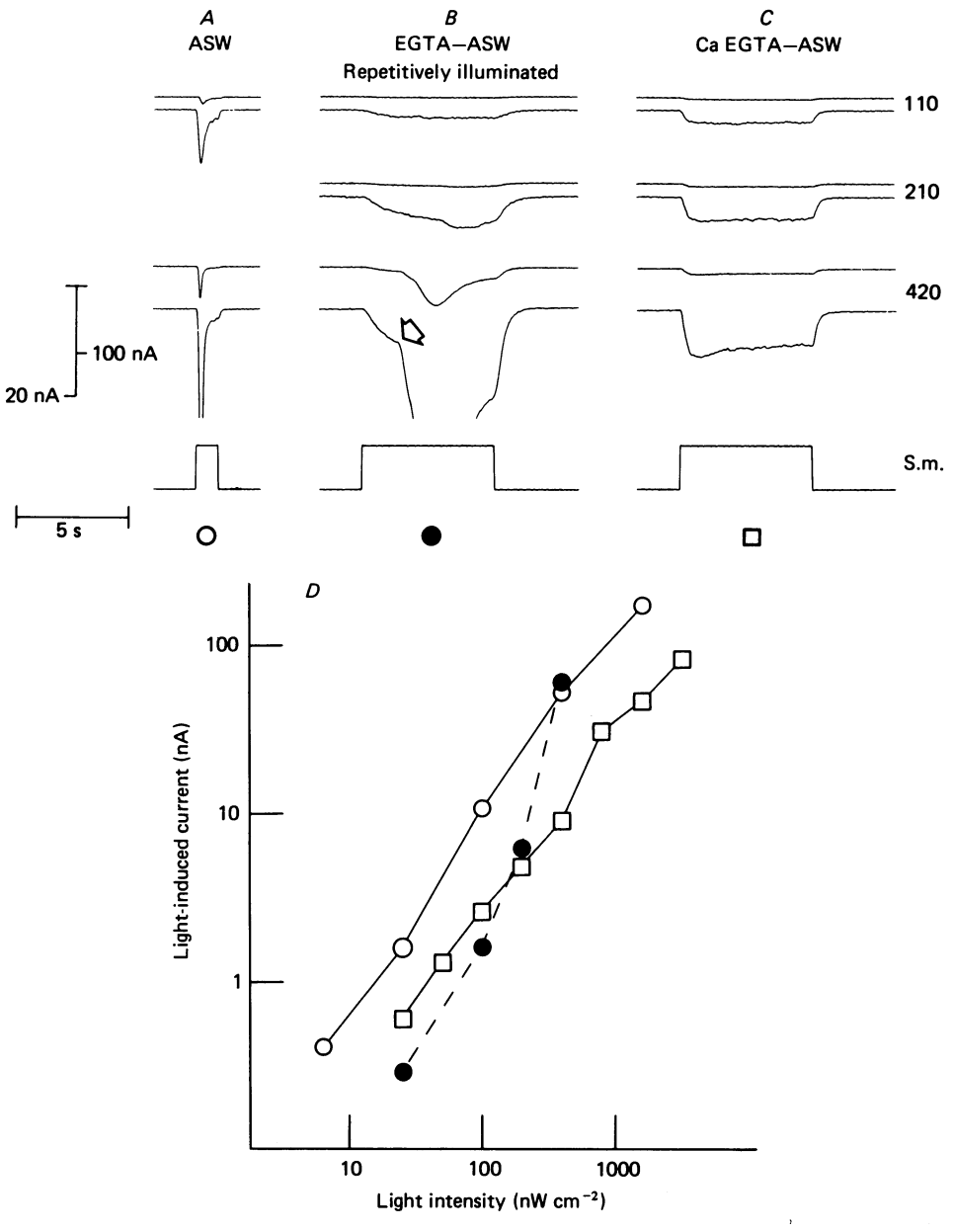

Fig. 2. Light-induced currents in EGTA–ASW. The cell was repetitively illuminated ($1{\cdot}05 \times 10^{-7}$ W cm^{-2}, one per 20 s) and allowed to dark adapt for 2 min before each of a series of flashes of increasing intensity (vertical columns). Light-induced current was recorded at two different gains. The figures to the right of the traces give the intensity in nW cm^{-2}. S.m.: stimulus monitor. The cell was voltage clamped to the resting voltage in ASW (−62 mV). *A*, the cell was bathed in ASW (1 s duration flashes). *B*, after 32 min in EGTA–ASW (6 s duration flashes). The open arrow indicates the onset of a delayed increase in light-induced current. *C*, after 6 min in Ca EGTA–ASW (6 s duration flashes). *D*, the peak current induced by each of the flashes in *A*, *B* and *C* is plotted as a function of light intensity. ○: in ASW. ●: in EGTA–ASW. □: in Ca EGTA–ASW.

When cells were bathed in EGTA–ASW for 45 min or more in darkness and then illuminated, the light responses were little different from the responses of cells bathed only for a few minutes in EGTA–ASW. However, as Fig. 1*A* illustrates, cells bathed in EGTA–ASW and illuminated repetitively with flashes of moderate intensity ($> 10^{-7}$ W cm^{-2}) generated responses that declined progressively from their peak amplitude for at least the next 20 min.

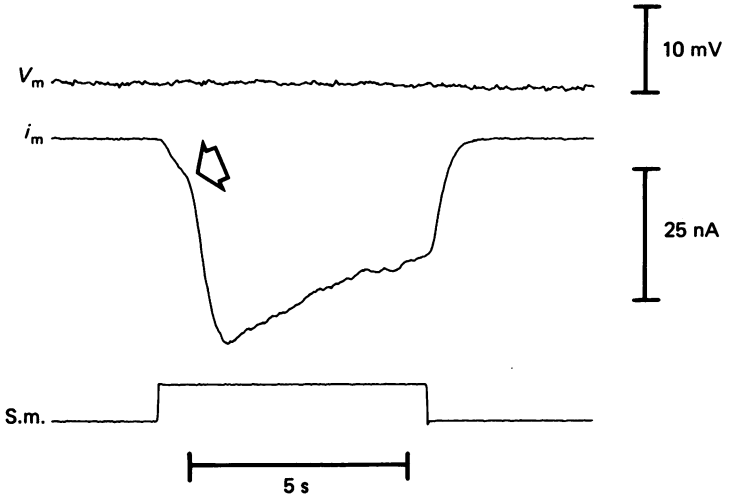

Fig. 3. Test of isopotentiality in voltage clamp. A photoreceptor was impaled with two KCl micropipettes and voltage clamped to the resting voltage in ASW (−57 mV). A third intracellular micropipette containing KOH–EGTA–Tris solution was used to measure the membrane voltage in another region of the cell. The cell was bathed in EGTA–ASW for 22 min and repetitively illuminated (35 flashes at $4{\cdot}2 \times 10^{-7}$ W cm^{-2}, 3 s duration, one flash per 20 s). After 10 min in darkness, the cell was illuminated with a series of flashes of increasing intensity. The response to a flash of intensity $8{\cdot}3 \times 10^{-7}$ W cm^{-2} is shown. During the delayed increase in light-induced current (i_m) (arrow), the membrane voltage (V_m) recorded by the third electrode did not change significantly; i.e. there was no failure of isopotentiality of intracellular voltage. S.m.: stimulus monitor.

After the decline in the amplitude of responses induced by repetitive stimulation in EGTA–ASW, some properties of the light responses differed from those of responses recorded from cells bathed either in ASW or in EGTA–ASW without repetitive stimulation. First, it has been found that when cells are bathed in ASW, the peak amplitude of the receptor potential increases smoothly in less than direct proportion to the intensity of the stimulus light (Lisman & Brown, 1975*a*). After cells were bathed in EGTA–ASW and repetitively illuminated, the amplitudes of the receptor potentials (Fig. 1*C*) increased abruptly in a narrow range of light intensity. This finding is similar to that reported by Stieve & Pflaum (1978). Currents induced by illumination of these cells were measured by a voltage clamp. As illustrated in Fig. 2*D*, the slope of the relationship between peak amplitude of the light-induced current and light intensity became much steeper than it had been in ASW. This increase in slope was observed in sixteen out of nineteen cells examined. Secondly, both receptor potentials and light-induced currents often showed a delayed increase from an initial level; this phenomenon occurred in fourteen out of nineteen cells and

is illustrated in several of the recordings displayed at fast sweep speeds. Each arrow on Figs. 1*B* and *C*, 2*B*, 3 and 4*B* indicates the onset of this delayed increase in response. The delayed increase of the light-induced current was not associated with a failure of the voltage clamp to maintain the cell isopotential. As Fig. 3 shows, there was no detectable change in intracellular voltage recorded by a third micropipette while the delayed increase in current occurred (two experiments).

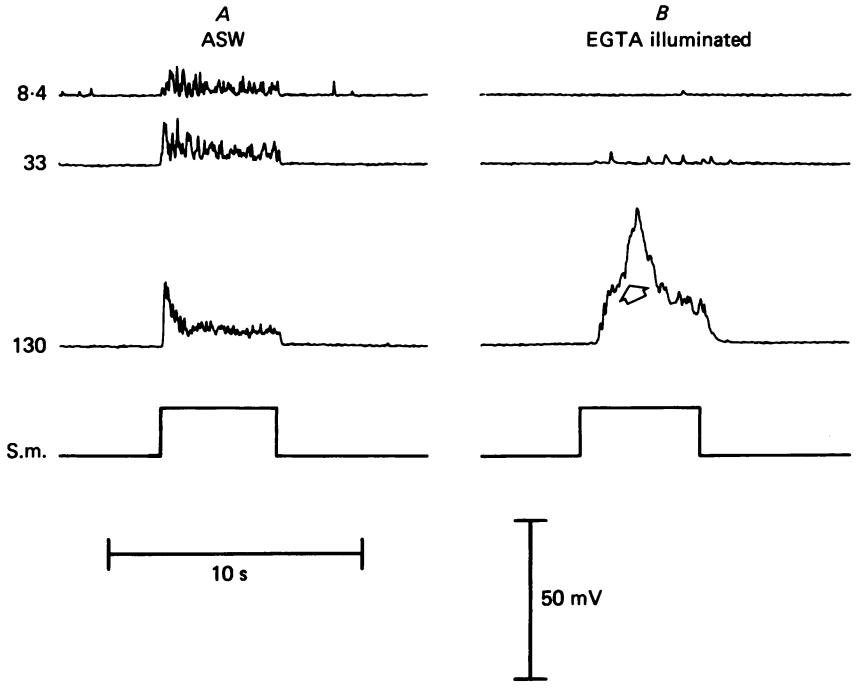

Fig. 4. Light responses in EGTA–ASW. Records from one of two cells that continued to produce discrete events after repetitive illumination in EGTA–ASW. Each vertical column shows receptor potentials elicited by a series of flashes of increasing intensity. Each series was begun after the cell was in darkness for 10 min. The figures to the left of the traces give the intensity in nW cm^{-2}. S.m.: stimulus monitor. *A*, in ASW. *B*, after 69 min bathed in EGTA–ASW during which the cell was repetitively illuminated (67 flashes at $1{\cdot}3 \times 10^{-4}$ W cm^{-2} followed by 32 flashes at $5{\cdot}3 \times 10^{-4}$ W cm^{-2}, 5 s duration, one flash per 20 s). The open arrow indicates the onset of a delayed increase in receptor potential amplitude.

For ten cells that had been bathed in EGTA–ASW and repetitively illuminated until the sensitivity became reduced, the responses elicited by dim illumination increased in amplitude when the bath was changed from EGTA–ASW to Ca EGTA–ASW (Fig. 2*D*). In Ca EGTA–ASW, the slope of the relationship between light-induced current and intensity was similar to that in ASW; moreover, no delayed increase in response was observed.

The light responses elicited by dim illumination of normal *Limulus* ventral photoreceptors are composed of the summation of discrete events (Millecchia & Mauro, 1969*a*; Wong, Knight & Dodge, 1982). In the majority of experiments in which cells were repetitively illuminated while bathed in EGTA–ASW, the light

responses elicited by dim illumination were not composed of recognizable discrete events. Fig. 4 shows records from one of the two cells in the nineteen tested that continued both to produce discrete events and to show the two characteristic phenomena: (1) a steeper slope of the relationship between light-induced current and intensity and (2) a delayed rise of the light response.

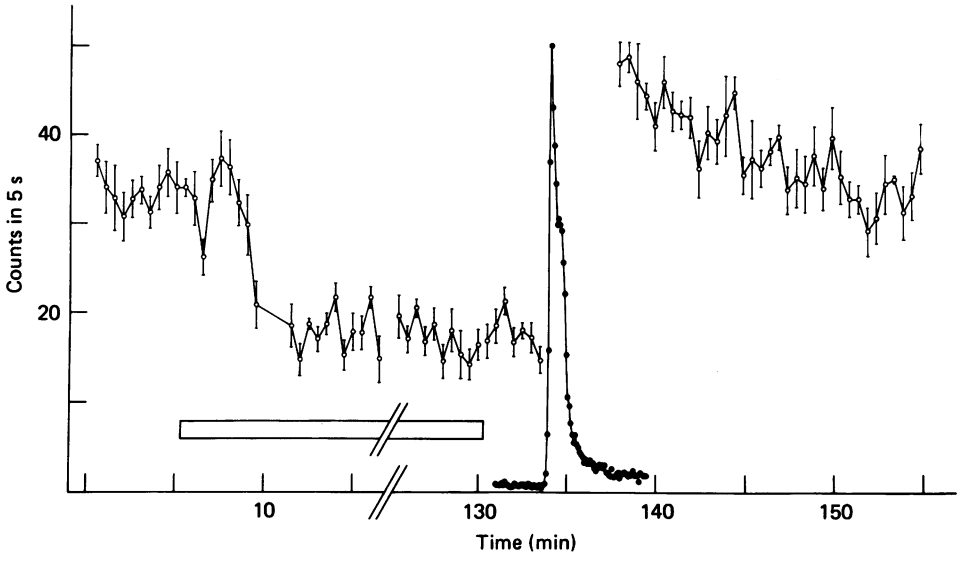

Fig. 5. Aequorin luminescence from a dark-adapted cell. Aequorin was injected into a single photoreceptor cell and the aequorin luminescence recorded while the cell was in the dark. The mean (±S.E. of mean) photon count in each 6 successive 5 s periods is plotted as a function of time. During the period indicated by the open bar, the cell was bathed in EGTA–ASW; at other times the cell was bathed in ASW. During the time period denoted 131–139 min, the count in each 5 s period is plotted without averaging as counts divided by 25. Note that resting luminescence is reduced during the EGTA–ASW bath. The remaining counts arose mainly from stray light and thermal noise in the photomultiplier tube. There was a large transient increase in luminescence after ASW was returned to the bath.

Fig. 6*E* illustrates that the behaviour of the cells returned nearly to normal when the bathing solution was changed back to ASW ($n = 6$). The changes in the light response described above occurred again if the bathing solution was changed to EGTA–ASW for a second time ($n = 3$).

Aequorin experiments

Photoreceptors were injected with aequorin and the luminescence was measured from dark-adapted cells. Fig. 5 shows the time course of luminescence from intracellular aequorin recorded while the bath was changed from ASW to EGTA–ASW and back. Within 10 min after the bath was changed to EGTA–ASW, the luminescence measured from the cell in darkness fell to a level not significantly different from the background. The luminescence remained at this low level as long as the cell was bathed in EGTA–ASW (seven cells). When the cell was returned to ASW in darkness,

the aequorin luminescence increased transiently and then declined to a level similar to that recorded before the EGTA–ASW bath (five cells).

A transient increase of aequorin luminescence (the aequorin response, described by Brown & Blinks, 1974) was elicited by a brief, bright flash delivered 10 min after the change to EGTA–ASW; the amplitude of this aequorin response was relatively

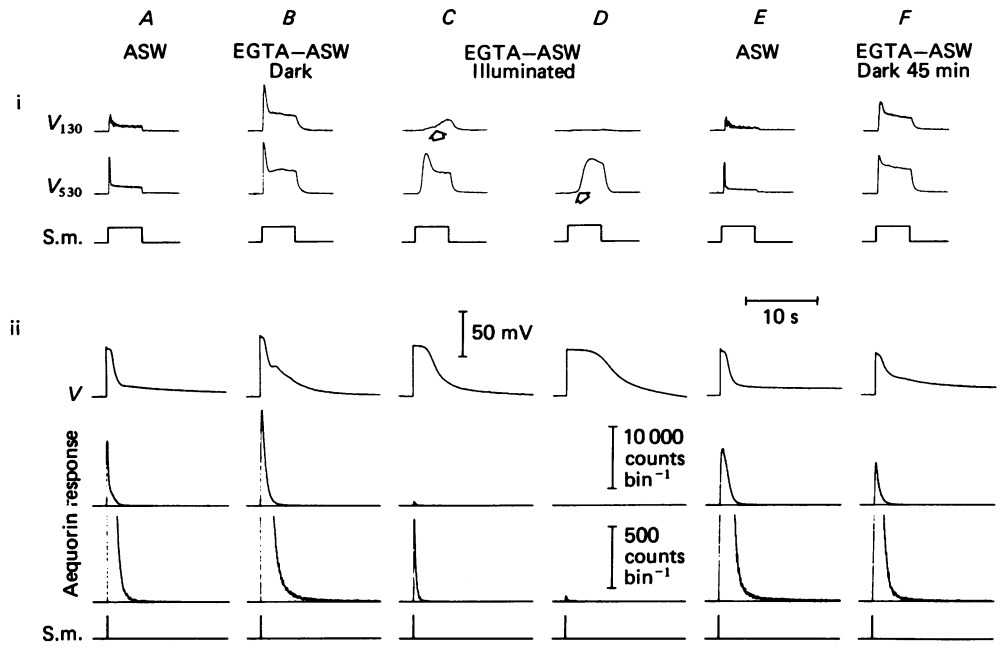

Fig. 6. Appearance of the delayed rising phase of the receptor potential correlates with a reduction of the aequorin response. Same cell as in Fig. 5. (i) For each of *A–F*; the cell was maintained in darkness for 10 min and then illuminated by a series of flashes of increasing intensity. The receptor potentials elicited by flashes of intensity 130 nW cm^{-2} (V_{130}) and by flashes of intensity 530 nW cm^{-2} (V_{530}) are shown. S.m.: stimulus monitor. Open arrows indicate the onset of a delayed increase in receptor potential amplitude. (ii) Voltage (*V*) and aequorin responses elicited by bright flashes (intensity $3{\cdot}3 \times 10^{-2}$ W cm^{-2}, duration 48 ms) delivered after 10 min in the dark. The photon count in successive 50 ms periods ('bins') is plotted as a function of time on two scales. S.m.: stimulus monitor. *A*, cell bathed in ASW. *B*, after 24 min in EGTA–ASW in darkness. *C*, after 64 min in EGTA–ASW during which the cell was repetitively illuminated for 11 min (33 flashes at $1{\cdot}3 \times 10^{-4}$ W cm^{-2}, 5 s duration, one flash per 20 s). *D*, after 115 min in EGTA–ASW during which the cell was further illuminated (for 8 min; 26 flashes as above). *E*, 26 min after return to ASW. *F*, after 45 min in EGTA–ASW in darkness.

unchanged compared to that recorded while the cell was in ASW, as shown in Fig. 6*B*. In five cells, the aequorin response was 110 ± 50 % of the control response. A comparison of Fig. 6*E* and *F* illustrates that a brief, bright flash evoked a relatively normal aequorin response (78 and 50 % of the ASW control, $n = 2$) from cells that were maintained in EGTA–ASW in darkness for 45–60 min. However, Fig. 6(*B*, *C* and *D*) also illustrates that a cell illuminated repetitively while bathed in EGTA–ASW generated aequorin responses that progressively declined in amplitude (to 2 ± 1 % of

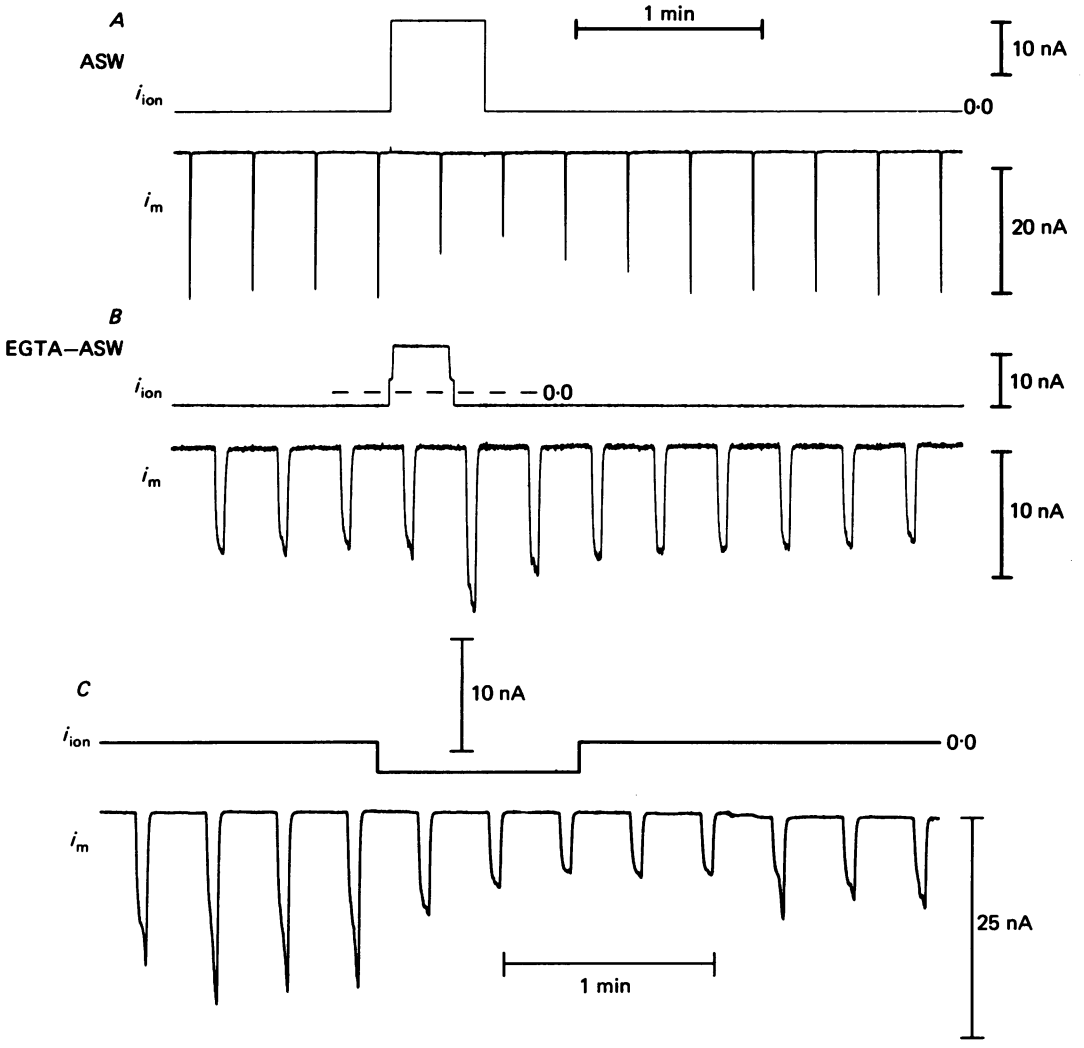

Fig. 7. Intracellular ionophoretic injections. *Injection of* Ca^{2+}. The effect of ionophoretic injection of Ca^{2+} on the light-induced current (i_m) was measured while the cell was bathed: *A*, in ASW and *B*, EGTA–ASW after a prolonged bath in EGTA–ASW during repetitive illumination. The photoreceptor cell was voltage clamped to the resting voltage in ASW (−61 mV). A third intracellular micropipette containing Ca $(OH)_2$–EGTA–Tris solution was used to inject Ca^{2+} by ionophoresis. *A*, the cell was bathed in ASW and repetitively illuminated (300 ms flashes; intensity $1{\cdot}7 \times 10^{-6}$ W cm^{-2}; one flash per 20 s). The injection is marked by an upward deflexion of the ionophoresis current trace (i_{ion}). No current flowed through the ionophoresis pipette at all other times. *B*, the cell was bathed in EGTA–ASW for 17 min during which there was repetitive illumination (3 s flashes; intensity $2{\cdot}1 \times 10^{-7}$ W cm^{-2}; one flash per 20 s). The injection is marked by an upward deflexion of i_{ion}. At other times a small negative current was passed through the ionophoresis pipette to reduce leakage of Ca^{2+} into the cytosol. *Injection of EGTA*. The effect of ionophoretic injection of EGTA on the light-induced current (i_m) was measured (*C*) after repetitive illumination in EGTA–ASW. The cell was voltage clamped to its resting voltage in ASW (−48 mV). A third intracellular micropipette containing KOH–EGTA–Tris solution was used to inject EGTA by ionophoresis. The cell was bathed in EGTA–ASW for 24 min and repetitively illuminated ($4{\cdot}2 \times 10^{-7}$ W cm^{-2}, 3 s duration, one flash per 20 s). The injection is marked by a downward deflexion of i_{ion}. No current flowed through the ionophoresis pipette at other times.

the control amplitude, $n = 7$). When the cell was returned to ASW, the aequorin response recovered to a relatively normal amplitude ($86 \pm 29\%$ of original response in ASW, $n = 5$) although the time course of the response was sometimes slowed (two out of five cells) as shown in the example in Fig. 6*E*.

Ionophoretic injections

Lisman & Brown (1972) reported that the light-induced current of ventral photoreceptors bathed in ASW was transiently reduced when Ca^{2+} was injected by ionophoresis from an intracellular micropipette containing a Ca-EGTA–Tris solution. This finding is illustrated in Fig. 7*A* for a cell bathed in ASW. Fig. 7*B* shows that after this cell was repetitively illuminated in EGTA–ASW, the light-induced current transiently increased when Ca^{2+} was injected. This Ca^{2+}-induced increase in the size of the light-induced current (by $77 \pm 18\%$) was observed during twenty-one ionophoretic injections of Ca^{2+} into seven cells. The average injection was $2{\cdot}3 \times 10^{-7}$ C. Control injections of K^+ were made from micro-electrodes containing a KOH–EGTA–Tris solution. K^+ injections that were about 6 times larger than the Ca^{2+} injections (average $1{\cdot}3 \times 10^{-6}$ C) increased the light-induced current by $3 \pm 1\%$.

Fig. 7*C* illustrates that the light-induced current from cells that were bathed in EGTA–ASW and repetitively illuminated was reduced (by $63 \pm 10\%$, $n = 5$) when EGTA was injected by ionophoresis. This decrease in the size of the light-induced current was only partially reversible.

Measurement of total cell Ca^{2+} by atomic absorption

The total intracellular Ca of end-organs ($n = 12$) bathed in ASW in darkness was measured to be 36 ± 25 pmol Ca μg protein^{-1}. The average total intracellular Ca was not significantly affected by bathing end-organs in EGTA–ASW for 30 min. Repetitive illumination of end-organs bathed in EGTA–ASW did not significantly change the average total intracellular Ca (Table 1).

TABLE 1. Total intracellular Ca: effect of bathing end-organs in EGTA–ASW. The total intracellular Ca per unit protein in a whole end-organ bathed in EGTA–ASW is expressed as a percentage of the intracellular Ca in the second end-organ from the same animal bathed in ASW. None of the values is significantly different from 1·0, using a one-tailed *t* test

Treatment	Intracellular Ca experimental/control
30 min in EGTA–ASW, all experiments	$0{\cdot}79 \pm 0{\cdot}12$, $n = 11$
30 min in EGTA–ASW, in darkness	$0{\cdot}73 \pm 0{\cdot}16$, $n = 6$
30 min in EGTA–ASW, illuminated	$0{\cdot}87 \pm 0{\cdot}20$, $n = 5$

DISCUSSION

Lisman & Brown (1972) proposed that an increase in $[Ca^{2+}]_i$ may be a step in the adaptation process. The decrease in steady-state aequorin luminescence indicates that $[Ca^{2+}]_i$ decreased while the cells were bathed in EGTA–ASW. Both the initial transient and the plateau of the receptor potential increased in amplitude. This result is compatible with the proposal that an increase of $[Ca^{2+}]_i$ acts to attenuate the light response (Lisman & Brown, 1972).

Brown & Blinks (1974) interpreted the aequorin response measured in the absence of extracellular Ca^{2+} as a release of Ca^{2+} into the cytosol from a light-labile intracellular store. Our results indicate that a considerable light-labile store remains after a prolonged bath in EGTA–ASW in darkness. However, the aequorin response declines progressively when cells are repetitively illuminated. This result supports similar findings of Maaz & Stieve (1980) that were made with Arsenazo III. After repetitive illumination either the light-labile store becomes depleted or the store no longer releases Ca^{2+}. Measurement of total intracellular Ca by atomic absorption spectroscopy showed that this did not change significantly during repetitive illumination in EGTA–ASW. If the light-labile store comprised only a small fraction of total cell Ca then depletion of this store might not significantly affect the measured total cell Ca.

We do not believe that a change of $[Ca^{2+}]_i$ is a sufficient and unique step that links photon absorption to the light-induced change in membrane conductance. Cells in which the light-induced change of $[Ca^{2+}]_i$ has been greatly reduced by injection of the Ca^{2+} buffer EGTA continue to respond to light (Lisman & Brown, 1975*b*). However, as suggested by Stieve & Bruns (1980), we presume that at least one step in the excitation mechanism requires cytosolic Ca^{2+}. Within this hypothetical framework, cytosolic Ca^{2+} is at a concentration that allows this step to proceed with close to maximum efficiency when cells are bathed in ASW. The efficiency of this step is reduced after repetitive illumination when extracellular Ca^{2+} is low. In this state, intracellular ionophoretic injection of Ca^{2+} can increase $[Ca^{2+}]_i$ enough to increase the efficiency of the excitatory mechanism and therefore increase the amplitude of responses elicited by dim stimuli. Also in this state, the Ca^{2+} that is released from the light-labile store by brighter illumination causes $[Ca^{2+}]_i$ to rise. As $[Ca^{2+}]_i$ rises, the efficiency of excitation increases and a greater amount of Ca^{2+} is released from the light-labile store. This positive feed-back mechanism causes both a delayed rising phase of the light response and a more than proportionate relationship between the amplitude of the light response and stimulus intensity.

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