

Pressure Injection of Calcium Both Excites and Adapts *Limulus* Ventral Photoreceptors

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ABSTRACT Single pressure injections of 1–2 mM calcium aspartate into the light-sensitive region of *Limulus* ventral photoreceptors resulted in a rapid, 20–40-mV depolarization lasting ~2 s. The depolarization closely followed the rise in intracellular free calcium caused by the injection, as indicated by aequorin luminescence. The depolarization was followed by reversible desensitization (adaptation) of responses to both light and inositol 1,4,5 trisphosphate. Similar single injections of calcium into the light-insensitive region of the receptor were essentially without effect, even though aequorin luminescence indicated a large, rapid rise in intracellular free calcium. The depolarization caused by injection of calcium arose from the activation of an inward current with rectification characteristics and a reversal potential between +10 and +20 mV that were similar to those of the light-activated conductance, which suggests that the same channels were activated by light and by calcium. The reversal potentials of the light- and calcium-activated currents shifted similarly when three-fourths of the extracellular sodium was replaced by sucrose, but were not affected by a similar replacement of sodium by lithium. The current activated by calcium was abolished by prior injection of a calcium buffer solution containing EGTA. The responses of the same cells to brief light flashes were slowed and diminished in amplitude, but were not abolished after the injection of calcium buffer. Light adaptation and prior injection of calcium diminished the calcium-activated current much less than they diminished the light-activated current.

INTRODUCTION

Illumination of *Limulus* ventral photoreceptors results in a depolarization that is caused by an increased membrane permeability to sodium ions (Millecchia and Mauro, 1969; Brown and Mote, 1974). Prolonged or bright illumination adapts the photoreceptor by reversibly reducing the light-activated membrane permeability (Millecchia and Mauro, 1969).

Bright flashes of light, which adapt the cell, rapidly increase the intracellular

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concentration of calcium ions, Ca_i (Brown and Blinks, 1974). The resulting transient rise in Ca_i can exceed $40 \mu M$ in amplitude, reach its peak in <300 ms, and decline to half-maximal amplitude in <2 s (Brown and Blinks, 1974; Brown et al., 1977; Nagy and Stieve, 1983; Levy and Fein, 1985). When the cell is not voltage-clamped, the steady level of Ca_i during prolonged illumination may depend on leakage of extracellular calcium into the cell (Stieve et al., 1984). However, the transient rise in Ca_i after a bright flash appears to result from a release of calcium from internal stores (Brown and Blinks, 1974; Levy and Fein, 1985) and is localized to the region of the cell that is most sensitive to light (Levy and Fein, 1985).

Lisman and Brown (1972) observed that prolonged ionophoretic injection of calcium reversibly desensitized the response of ventral photoreceptors to light. Using a voltage-clamp technique, Brown and Lisman (1975) were unable to detect any inward current associated with ionophoretic injection of calcium. These experiments led them to propose that a rise in intracellular calcium can cause photoreceptor adaptation, but not excitation (Brown and Lisman, 1975).

Recently, Fein et al. (1984) and Brown et al. (1984) have demonstrated that pressure injection of inositol 1,4,5 trisphosphate (IP_3) both excites and adapts ventral photoreceptors. IP_3 induces a rapid rise in Ca_i when it is injected into ventral photoreceptors, probably through the release of intracellular stores (Brown and Rubin, 1984; Corson et al., 1984). Adaptation of the photoreceptor through release of calcium by IP_3 is clearly compatible with the finding that a rise in Ca_i adapts the cell (Lisman and Brown, 1972), but excitation through the release of calcium was unexpected, given the negative result of Brown and Lisman (1975). However, the ionophoretic injections of calcium used by Brown and Lisman (1975) might have been insufficient to excite the cell. The ionophoretic injections of calcium may not have been localized to the light-sensitive region or they may have delivered calcium too slowly to overcome the cell's native buffering capacity sufficiently to produce a large, transient increase in free calcium. The localized action of IP_3 within the photoreceptor (Brown et al., 1984; Fein et al., 1984) and the rapidity of the release of calcium raise the possibility that IP_3 could generate localized calcium concentrations sufficiently high that it could excite the cell as well as adapt it.

Two recent technical advances have made it worthwhile to reinvestigate the effects of calcium injection. The revision of the morphology of the ventral photoreceptor (Stern et al., 1982; Calman and Chamberlain, 1982) now allows an unequivocal identification of the light-sensitive region of the photoreceptor. A technique for the rapid, localized injection of substances from glass micropipettes into ventral photoreceptors has also been developed (Corson and Fein, 1983). These technical advances have enabled us to investigate the effect of rapid, pulsed injections of millimolar concentrations of calcium into the light-sensitive region. We report that injections of this kind depolarize the photoreceptor before adapting it and that the depolarization appears to result from the activation of the light-sensitive conductance. The results suggest that release of calcium by IP_3 may be sufficient to explain its ability to excite and adapt ventral photoreceptors. Excitation by light, however, appears to be more complex and

calcium is more likely to act as a modulator of the release of another, unknown transmitter.

A brief account of these findings has appeared in abstract form (Payne et al., 1984).

METHODS

Recording and Stimulation

Conventional methods of recording and stimulation, described in detail elsewhere (Fein and Charlton, 1975, 1977), were used for intracellular recording and for voltage clamp of ventral nerve photoreceptors of *Limulus polyphemus*. Cells were stripped of glia in some experiments, according to the method of Stern et al. (1982), so as to expose the rhabdomeral (R) and arhabdomeral (A) lobes described by Stern et al. (1982). In other experiments, where it was not practical to strip the glia from the cell, the cell was scanned by light from a 10- μm -diam spot after impalement, to determine the proximity of the micropipettes to the most sensitive area of the cell.

Cells were stimulated by white light from a 45-W tungsten-halogen lamp delivering a maximum of 40 mW/cm² when focused onto the preparation. Stimulus energies are given in this article according to the log₁₀ attenuation from this maximum energy. Cells were dark-adapted for 20 min before being impaled under infrared illumination. Most cells exhibited spontaneous and light-evoked discrete waves of depolarization after impalement.

Rapid pressure injection of substances into the cells was achieved by applying brief pressure pulses to the back of a blunt micropipette that impaled the cell, according to the method of Corson and Fein (1983). In some experiments, the cell was impaled with two such pipettes, so as to inject either of two solutions.

Before the cell was impaled, ejections of solution were made into an oil droplet and the injected volume was estimated. Single injections delivered ~1 pl into the oil drop. This volume was used to estimate a volume of 1–10 pl injected into the cell, according to the method of Corson and Fein (1983).

Chemicals and Injection Solutions

All inorganic reagents used were of analytical grade. EGTA and HEPES were obtained from Sigma Chemical Co., St. Louis, MO. MOPS (3-[*N*-morpholino]propanesulfonic acid) was obtained from Boehringer-Mannheim Biochemicals, Indianapolis, IN. IP₃ was the generous gift of Drs. R. F. Irvine and M. J. Berridge. The method of preparation of the IP₃ was as described by Irvine et al. (1984).

Solutions of calcium aspartate or magnesium aspartate were made by dissolving appropriate amounts of Ca(OH)₂ or MgO in L-aspartic acid to make a solution of 100 mM Ca(aspartate)₂ or Mg(aspartate)₂. These solutions were then diluted with a carrier solution to the final concentration to be injected into the cell. The carrier solution contained 100 mM potassium aspartate and 10 mM HEPES, pH 7. Electrodes containing 1 or 2 mM calcium were found to clog after 15–20 min in a cell. Clogging usually occurred abruptly, making it impossible to inject the solution even when the pressure at the back of the pipette was increased. Reliable pressure injections over long periods were therefore impossible. However, we found that the addition of 0.001% Triton X-100 (Serva Brand, Feinbiochemica, Heidelberg, Federal Republic of Germany) to the injection solution reduced the chances of the electrodes clogging and extended the available experimental time. The injection of 0.001% Triton X-100 in carrier solution was found to have no significant effect on the cells. We thank Dr. S. L. Tamm for suggesting this procedure.

Aequorin was the generous gift of Dr. O. Shimomura. It was diluted to a final concentration of 7 mg/ml in carrier solution to which 100 μ M EGTA had been added to prevent discharge of the aequorin before injection. 10–100 pl of aequorin solution was injected into the cells by a series of pressure pulses as described above. Luminescence from the aequorin was collected with a long-working-length 40 \times objective (model M-Plan 40, Nikon Inc., Garden City, NY) and monitored with a photomultiplier (model 9658RA, Thorn-EMI, Plainview, NY) operated in conjunction with a photon counter (model 1770, PRA Inc., London, Ontario, Canada), which repetitively displayed the counts accumulated in 10-ms time bins.

Artificial Seawaters

The compositions of the artificial seawaters (ASWs) used in the experiments of this and the following article (Payne et al., 1986) are given in Table I. All salines had a pH of 7.0. The flow chamber used for the experiments that required changes of solution had a flow rate of 2.5 ml/min and a total dead volume of 1.3 ml.

TABLE I
Compositions of ASWs

	Na	Sucrose	Li	Ca	K	Mg	SO ₄	Cl	HEPES
	<i>mmol/liter</i>								
Normal	437	—	—	10	10	45	25	505	10
1 mM Ca	437	—	—	1	10	45	25	487	10
0.5 mM Ca	437	—	—	0.5	10	45	25	486	10
Sucrose	102	670	—	0.5	10	45	25	151	10
LiCl	102	—	335	0.5	10	45	25	486	10

RESULTS

The results of our investigation are grouped into the following main themes: (a) the similarities between the light- and calcium-activated conductances; (b) the specificity of the response to calcium injections; and (c) interactions among the effects of light, calcium, and IP₃.

Similarity Between the Light-activated and Calcium-activated Conductance

Injection of calcium into the R-lobe depolarizes and adapts the photoreceptor. Photoreceptors were stripped of their glia to expose their light-sensitive, rhabdomeral R-lobe and insensitive, arhabdomeral A-lobe (Stern et al., 1982; Calman and Chamberlain, 1982). Fig. 1A is a diagram of a stripped photoreceptor, which was subsequently impaled in its A-lobe with a micropipette containing 2 mM calcium aspartate dissolved in carrier solution (see Methods). The photoreceptor was illuminated every 12 s by diffuse light flashes. The injection of calcium into the A-lobe by a single 30-ms pressure pulse applied to the pipette had very little effect (Fig. 1C). The delivery of an estimated 1–10 pl of fluid was confirmed by viewing the disturbance of the receptor's cytoplasm during the injection (Corson and Fein, 1983). A sequence of three 30-ms pulses produced a small (<5 mV) depolarization and a small subsequent desensitization of the response to light.

The pipette was then removed from the A-lobe and placed in the R-lobe. A pressure pulse identical to that used for injections into the A-lobe induced a large transient depolarization of 30 mV amplitude and a substantial, reversible

desensitization of the response to light flashes (Fig. 1D). After confirmation of this result by a second injection, the receptor was dark-adapted and the sensitivity at the two injection sites was determined using a dim 10- μm -diam spot of light. The sensitivity to light at the R-lobe injection site was found to be 40-fold higher than at the A-lobe injection site (Fig. 1B). The results shown in Fig. 1 were confirmed in seven other cells. The peak amplitudes of depolarizations owing to

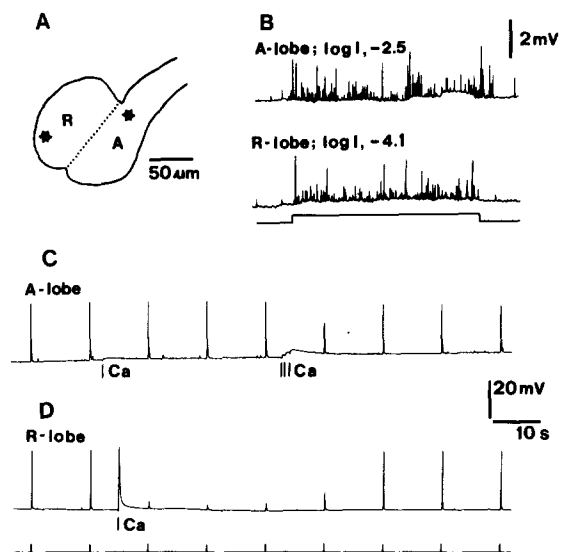


FIGURE 1. Localized effects of pressure injection of calcium into a ventral photoreceptor. (A) Diagram of a stripped photoreceptor traced from the video monitor: A = A-lobe, R = R-lobe. The axon originates at the top right-hand corner of the diagram. The dotted line shows the site of a large change in sensitivity to light, which is probably associated with the boundary between the lobes. The stars show the position of cytoplasmic disturbance caused by the injections (Corson and Fein, 1983). (B) Discrete waves elicited by a 10- μm light spot centered on the injection sites in the A-lobe (upper trace) and the R-lobe (middle trace). The lower trace indicates the stimulus duration. The log of the light intensity (I) at each site is indicated above the traces. The A-lobe was 1.6 \log_{10} units less sensitive than the R-lobe. The same time scale was used for C and D. (C and D) Responses to 1–10-pI (30 ms; 20 psi) injections into the A-lobe (C) and subsequently into the R-lobe (D) of 2 mM calcium aspartate in carrier solution. The bars below the traces indicate the times of the injections. Diffuse light flashes (duration, 10 ms; $\log_{10} [I] = -4$) were delivered at the times indicated by the bottom trace.

single calcium injections into the R-lobe were typically 20–40 mV, reaching their peak between 150 and 500 ms after the injection began. Subsequent light responses were transiently reduced 3–10-fold in amplitude.

Ionophoretic injection of calcium into calcium-depleted ventral photoreceptors produces a facilitation of the light response (Bolsover and Brown, 1982). We have not yet attempted to repeat Bolsover and Brown's result by bathing the cells in EGTA before pressure injection of calcium. We have not seen facilitation

of the response to light after pressure injection of 1 or 2 mM calcium into cells bathed in normal ASW. Responses to light are either adapted or unaffected by the injection, depending on the position of the electrode.

The voltage dependence of the calcium-activated current is similar to that of the light-activated current. The depolarization caused by the injection of calcium

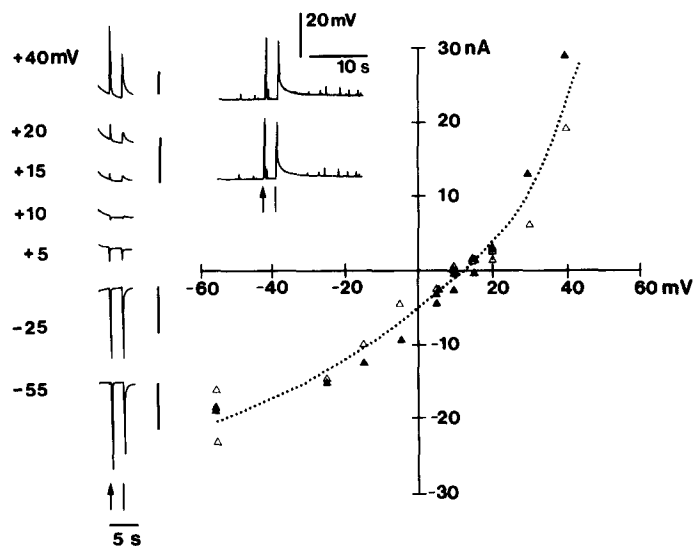


FIGURE 2. Graph of peak current vs. transmembrane potential for currents activated by light (solid symbols) or calcium injection (open symbols). The dotted line is the solution to Eqs. 1 and 2 in the text, using $E_{rev} = +12$ mV, $a = 0.42$ μ S, $b = 0.0022$ μ S/mV, and $c = 0.0060$ μ S. Cells were impaled with one micropipette containing 3 M KCl and one containing 2 mM calcium in carrier solution with 0.001% Triton X-100. The voltage clamp was imposed by passing current through the KCl pipette while potential was recorded with the other. The cell's potential was held at its resting value (-55 mV). Command pulses were delivered to the clamp to depolarize the cell in steps of up to 95 mV. These potential steps were of 8 s duration. 2 s after the onset of the step, a sample-and-hold device was used to subtract the steady outward current. A flash of light (duration, 10 ms; $\log_{10} [I] = -3.5$) was then delivered 2 s later, followed by a 1–10-pl injection (50 ms; 30 psi) from the electrode containing calcium. Records of the responses to light and calcium at the potentials indicated are shown to the left of the graph. The arrow and the bar below the traces indicate the times of the light and the calcium injection, respectively. The vertical scale markers represent 10 nA. The records inset into the graph show voltage records, obtained through both micropipettes, of the response to a light flash and a calcium injection.

arises from a calcium-activated inward current. This current is shown in the voltage-clamp experiment illustrated in Fig. 2. We compared the dependence on membrane potential of the calcium-activated and light-activated currents. Cells were impaled with two micropipettes. One micropipette, placed in the most sensitive area of the cell, contained 1 or 2 mM calcium aspartate in carrier solution. Because the experiments demanded repeated, reliable injections of 2

mM calcium over long periods of time, we included 0.001% Triton X-100 in this pipette to prevent clogging (see Methods). The other, sharper electrode impaling the cell contained 3 M KCl. Control injections of 0.001% Triton X-100 in carrier solution into 16 cells were without effect on 12 cells and produced small, irreversible desensitizations (<0.3 log units) in 4 cells. Membrane voltage was recorded through the pipette containing calcium and current was passed through the KCl electrode to voltage-clamp the receptor.

We were careful to account for artifacts induced by injection of calcium through the voltage electrode, since these would distort the current record under clamp. At the beginning and end of the experiment, therefore, we recorded the voltage change through both the voltage electrode and the KCl electrode that was associated with a calcium injection. The inset in the graph of Fig. 2 shows that the potentials recorded by the electrodes during and after the injection differed by <1 mV, so injection artifacts appear to be negligible. This conclusion is also supported by the experiment of Fig. 5, in which a similarly voltage-clamped cell showed only a very small artifact owing to calcium injection after the injection of calcium buffer.

After the size of the injection artifact was checked, the photoreceptor was voltage-clamped to its resting potential. The responses to a flash of light and to a subsequent calcium injection were recorded at the resting potential and 4 s after changing the clamp potential to values between -25 and +40 mV (Fig. 2). After each injection, the sensitivity of the photoreceptor to light was allowed to recover. When photoreceptors were voltage-clamped to their resting potential, 1-10- μ l injections of 2 mM calcium into the most sensitive region of the photoreceptors elicited currents of 10-20 nA amplitude. Fig. 2 illustrates records of light- and calcium-activated currents at a variety of membrane potentials. Note the pronounced slowing of the calcium-activated current at potentials more positive than the reversal potential. This slowing was consistently observed in five cells investigated. A similar slowing of the light response was not consistently observed, although slowing is clearly shown in the records of Millecchia and Mauro (1969, Fig. 5), in which more intense flashes than those of our experiments were used. The variability of the time course of individual responses to IP₃ (Fein et al., 1984) makes it impossible to state whether a similar slowing of the inward currents induced by IP₃ occurs.

The peak amplitudes of the calcium-activated current and the light-activated current are plotted against membrane potential in Fig. 2. The graph of Fig. 2 demonstrates that the light-activated current and the calcium-activated current have similar reversal potentials and are similarly rectified. The dotted lines through the points in Figs. 2 and 3 are solutions to Eqs. 1 and 2, which were used by Millecchia and Mauro (1969) to empirically describe the light-sensitive conductance, g_L :

$$g_L = a + bV + c \exp(0.1V); \quad (1)$$

$$i_L = g_L(E - E_{rev}), \quad (2)$$

where i_L is the light-sensitive current, E_{rev} is the reversal potential of the light-sensitive current, E is the membrane potential, and a , b , and c are constants

chosen to best fit the data. The values of a , b , and c have a ratio of their magnitudes similar to those used by Millecchia and Mauro (1969, Fig. 8) to describe the current-voltage relationship of the discrete waves of inward current evoked by single photons. In Figs. 2 and 3, the ratio $a:b:c$ is 1:0.0052:0.0143. Millecchia and Mauro (1969) used a ratio of 1:0.0055:0.0178.

Fig. 3 shows normalized current-voltage relations from five cells injected with either 1 mM calcium aspartate in carrier solution or 2 mM calcium aspartate in

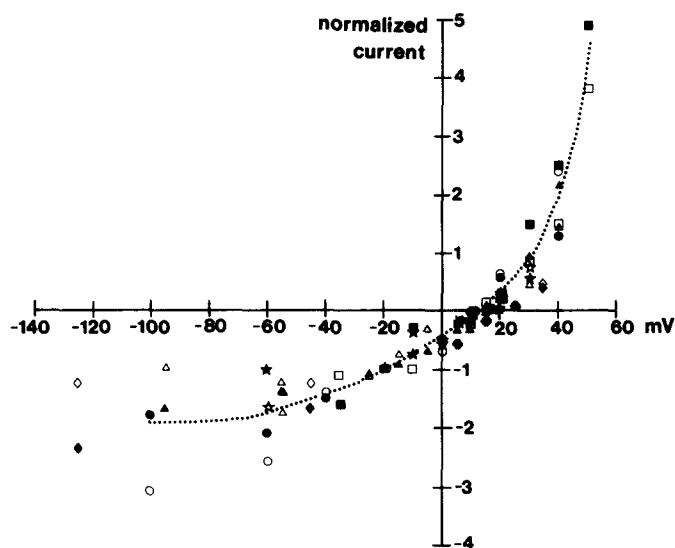


FIGURE 3. Normalized current-voltage relationships of light- and calcium-activated currents. Peak normalized amplitude of currents activated by light (solid symbols) or 1–10-pl injections of calcium (open symbols) are plotted against potential. Three photoreceptors were injected with 1 mM calcium aspartate in carrier solution (\star , \diamond , \circ). Two photoreceptors were injected with 2 mM calcium aspartate in carrier solution containing 0.001% Triton X-100 (Δ , \square). The peak amplitudes of the responses to light or calcium were normalized to their value at -20 mV. For experimental details, see text and the legend to Fig. 2. The dotted line is the solution of Eqs. 1 and 2 using $E_{rev} = 12$ mV, $a = 0.035$, $b = 0.00018$ mV $^{-1}$, $c = 0.00050$.

carrier solution containing 0.001% Triton X-100. The data demonstrate that the calcium- and light-activated currents are similar in reversal potential and rectification. The similarity of the data from the different cells demonstrates that calcium activates a conductance that is well described by Eqs. 1 and 2 with a reversal potential between $+10$ and $+20$ mV.

The calcium-activated conductance selects for sodium and lithium ions. At physiological extracellular sodium concentrations (Na_o), the light-induced conductance is carried primarily by sodium ions. Brown and Mote (1974) found that the reversal potential, E_{rev} , of the light-activated current changed by ~ 55 mV per decade change in Na_o , for $Na_o > 212$ mM. At lower concentrations of Na_o , the slope of this relationship declined in most cells, presumably because of a

substantial conductance to other ions, such as potassium. Thus, for a reduction of Na_o from 425 to 106 mM, Brown and Mote reported reductions in E_{rev} of between 20 and 33 mV, rather than the 35 mV expected from the Nernst equation if sodium were the only permeable ion. The replacement of sodium by lithium, however, had no effect on E_{rev} , which suggests that the channel has a similar permeability to lithium and sodium. Brown and Mote (1974) also reported that the replacement of sodium by sucrose or lithium tended to desensitize the photoreceptor. This desensitization could be reduced by the reduction of the calcium concentration in the bathing medium. The experiments that follow were therefore performed using ASW containing 0.5 mM calcium.

To determine the effect of lowering Na_o on the calcium-activated current, we impaled cells with two electrodes, one containing 3 M KCl, and the other containing 2 mM calcium and 0.001% Triton X-100 in carrier solution. The calcium-containing electrode was placed in the cell's most sensitive region. The cell was then voltage-clamped and the reversal potentials of the light- and calcium-activated currents were determined as in the experiments of Figs. 2 and 3. The ASW bathing the cell was then switched to one in which the sodium concentration was reduced from 437 to 102 mM by replacement with sucrose or lithium (see Methods). After equilibration, the reversal potentials of the light- and calcium-activated currents were measured once more.

Fig. 4 illustrates experiments on two cells. In the first experiment, sodium was replaced by sucrose, resulting in 33- and 35-mV reductions in E_{rev} for the light- and calcium-activated conductances, respectively. Experiments on two other cells showed shifts of 28 and 26.5 mV (cell 1) and 25 and 23 mV (cell 2) in the respective reversal potentials. The small differences in the reversal potentials of the light- and calcium-activated currents observed after replacement of sodium (0, 2.5, and 1.5 mV for the three cells, respectively) were similar to the 0–4-mV range of differences in the reversal potentials encountered in a given cell in normal ASW (for examples, see Figs. 2 and 3). A similar replacement of sodium with lithium had no significant effect on E_{rev} for both the calcium- and light-activated currents recorded in three other cells. An example is illustrated in Fig. 4.

Because the reversal potentials of the light- and calcium-activated currents were similar in normal ASW and when sucrose or lithium replaced approximately three-fourths of the sodium in the bathing ASW, we conclude that the two underlying conductances are similarly permeable to sodium and lithium in the ASWs investigated. Our findings on the changes in the reversal potential of the light-sensitive current confirm those of Brown and Mote (1974).

Specificity of the Voltage-Clamped Response to a Calcium Injection

Injection of calcium buffer abolishes excitation and adaptation by subsequent injections of calcium. Because of the extreme dependence of the amplitude of the depolarization on the location of the calcium injection (Fig. 1), we sought a method for determining the specificity of the response other than the injection of control solutions. We decided to determine whether the prior injection of a calcium chelator would abolish the response to a calcium injection. We therefore impaled a cell with two blunt pipettes, one containing an EGTA solution designed to buffer free calcium, and the other containing 2 mM calcium aspartate dissolved

in carrier solution. The electrode containing calcium was inserted into the most light-sensitive region of the photoreceptor. We expected that the injected EGTA and CaEGTA would diffuse rapidly through the cytosol (Hellam and Podolsky, 1969). Therefore, the pipette containing EGTA was placed in the A-lobe of the photoreceptor. The calcium buffer solution contained 20 mM calcium, 100 mM K_2EGTA , and 500 mM KMOPS, pH 7. The concentrations of calcium, EGTA,

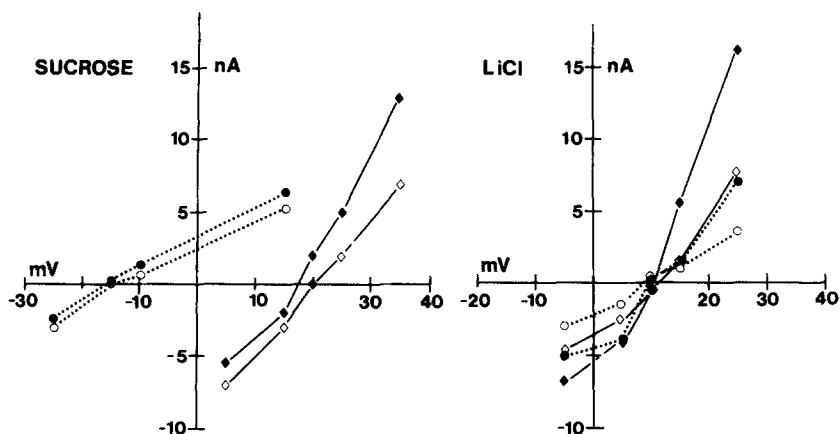


FIGURE 4. Effect of sodium replacement on the reversal potential of light- and calcium-activated currents. (Left) Peak current elicited by light flashes (solid symbols) and 1–10-pl injections of 2 mM calcium in carrier solution containing 0.001% Triton X-100 (open symbols). The diamonds show control current-voltage relationships when the cells were bathed in 0.5 mM calcium ASW. The circles show the same relationships 3 min after switching to sucrose ASW, in which the sodium concentration was reduced from 437 to 102 mM by 2 mol-for-mol replacement of NaCl with sucrose. $\text{Log}_{10}(I) = -3.5$; duration, 10 ms. Injection duration, 60 ms; pressure, 30 psi. (Right) The result of a similar experiment on another cell, in which sodium was reduced to 102 mM by replacing NaCl with LiCl. The diamonds show current-voltage relationships of the peak current activated by light (solid symbols) or calcium (open symbols) when the cells were bathed in 0.5 mM calcium ASW. The circles show the same relationships 5 min after switching to LiCl ASW in which the sodium concentration was reduced from 437 to 102 mM by equimolar replacement of NaCl with LiCl. $\text{Log}_{10}(I) = -3.5$; duration, 10 ms. Injection pressure, 30 psi; duration, 50 ms. For experimental details, see text and the legend to Fig. 2.

and MOPS were chosen so as to establish a free calcium concentration in the buffer solution of 0.1 μM and to maximize the area under the light response after injection (Payne and Fein, 1984). Because the photoreceptor was impaled with two electrodes, it could be voltage-clamped to its resting potential throughout the experiment.

Fig. 5A demonstrates that the injection of an estimated 1–10 pl of 2 mM calcium induced an inward current, as did repetitive flashes of light. The calcium-activated current began to rise during the pressure pulse and peaked 150 ms after the injection began. The response to light was reduced after the calcium injection and recovered 40 s later. 10–100 pl of calcium buffer solution was then

injected by a series of pressure pulses through the other pipette. Fig. 5B shows that a subsequent injection of calcium neither activated an inward current nor desensitized the response to light. This result is consistent with the interpretation that excitation and adaptation of the photoreceptor by the injection of calcium aspartate are due to a rise in Ca_i .

A comparison of the first responses to light in Fig. 5, A and B, shows that the injection of EGTA slowed and diminished the light response but did not abolish

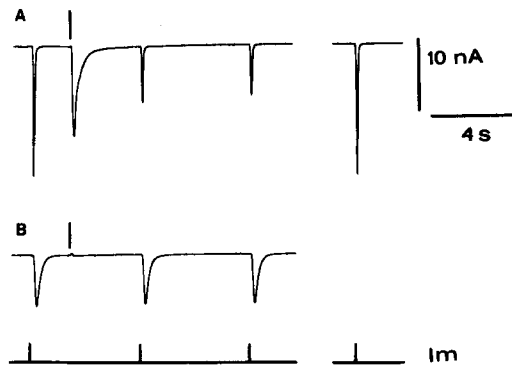


FIGURE 5. Abolition of the effects of calcium injection by intracellular EGTA. The cell was impaled with two electrodes. One contained 2 mM calcium in carrier solution, and the other contained 100 mM EGTA, 20 mM $Ca(OH)_2$, and 500 mM MOPS neutralized with KOH. The cell was voltage-clamped using the calcium aspartate pipette to record potential and the other pipette to pass current. The cell's potential was held at its resting value in darkness. (A) Inward currents elicited by diffuse light flashes (duration, 10 ms; $\log_{10} [I] = -3$) and by a 1–10-pl injection of calcium (100 ms; 30 psi). The gap in the record represents 40 s for recovery of the cell's response to light. The times of delivery of the flashes are given by the light monitor (lm) trace. The bars above the traces indicate the times of the injections. (B) Responses to the same intensity of flash and to the same pressure injection of calcium 50 s after pressure injection of 10–40 pl of calcium buffer through the other electrode. In the interval between traces A and B, <100 nC of (negative) charge entered the cell through the pipette containing the EGTA. This charge would raise intracellular EGTA by <1 mM, even if all the charge were carried by EGTA ions.

it, which is consistent with previous findings (Lisman and Brown, 1975). The result of Fig. 5 was confirmed in a total of seven cells.

Injection of magnesium, strontium, and barium. We tested whether magnesium, strontium, or barium could substitute for calcium in our injection experiments. Cells were impaled in their most sensitive region with electrodes containing 2 mM calcium, barium, magnesium, or strontium, using either chloride or aspartate as the anion (Table II). Flashes were delivered to the cells every 10 s. Table II shows the peak depolarization and desensitization produced by single 1–10-pl injections. 2 mM calcium or strontium, but not barium or magnesium, was able to transiently depolarize the photoreceptor. Injections of calcium, strontium, and barium, but not magnesium, reversibly desensitized the photo-

receptor's response to light. Thus, of the divalent ions tested, 2 mM strontium was the only one able to qualitatively reproduce the effects of calcium injection.

We noted in these and other control experiments that if the electrode remained in the ASW bathing the photoreceptor for several minutes before impalement, then the first one or two injections did induce small, rapid inward currents of <5 nA amplitude. Subsequent injections failed to produce any effect. We tentatively suggest that these effects result from contamination of the pipette tip by the 10 mM calcium in the ASW and note that clearing the pipette tip by a series of pressure pulses delivered before impalement of the cell greatly reduced the incidence of these depolarizations.

The time course of the calcium-induced depolarization closely follows the transient rise in Ca_i . Six photoreceptors were impaled with two micropipettes, one containing 2 mM calcium aspartate in carrier solution, and the other containing a

TABLE II
Effects of Injection of Divalent Ions

	Ca* (n = 5)	Sr* (n = 5)	Ba* (n = 4)	Mg [‡] (n = 8)
Depolarization (mV)	27±9	11±2	— [§]	— [§]
Desensitization [†] (%)	47±11	39±15	48±21	0.5±15

* Delivered as 2 mM chloride salt dissolved in carrier solution.

‡ Delivered as 2 mM aspartate salt dissolved in carrier solution.

§ Depolarization of <2 mV.

† Desensitization expressed as $(V_b - V_a)/V_b$ percent, where V_a is the amplitude of the response to a test flash 5 s after injection of divalent ion and V_b is the response before injection. The test flash was attenuated 3.5 log units; its duration was 10 ms.

solution of the luminescent photoprotein aequorin (Shimomura et al., 1962; Brown and Blinks, 1974). The photoreceptors were first pressure-injected with aequorin and then impaled with the calcium-containing electrode. Upon injection of 1–10 pl of calcium solution, records of aequorin luminescence similar to those of Fig. 6 were obtained from all the cells. As described above, the magnitude of the accompanying depolarization varied greatly from cell to cell, depending on the position of the electrode. Fig. 6 shows two extreme examples. In Fig. 6A, calcium was delivered into the region of the photoreceptor most sensitive to light, and a large, transient depolarization followed the aequorin luminescence after a small delay. In Fig. 6B, another photoreceptor was impaled with the electrode that contained calcium in a region that was insensitive to light. Fig. 6B demonstrates that the injection of calcium into this region produced a transient rise in aequorin luminescence similar to that shown in Fig. 6A. However, as expected from the experiment on stripped photoreceptors, there was no accompanying depolarization. The reason for the differing sensitivity to calcium injection of the A-lobe and R-lobe (Fig. 1) would therefore appear to be a lack of binding sites for calcium in the A-lobe rather than a difference in the magnitude or time course of the rise in Ca_i in the two lobes. For the other photoreceptors studied, the delay between the peak of the aequorin luminescence and the light response ranged from tens to hundreds of milliseconds. Longer delays were associated with depolarizations smaller than those of Fig. 6A.

Interactions among the Effects of Light, Injection of Calcium, and Injection of IP₃

Calcium injection desensitizes the photoreceptor's response to injections of IP₃. Pressure injection of IP₃ excites ventral photoreceptors (Fein et al., 1984; Brown et al., 1984). Like the response to dim light flashes, the response to IP₃ can be desensitized by bright or prolonged illumination, or by prior injection of IP₃. We wished to determine whether the adaptation of the response to IP₃ could be

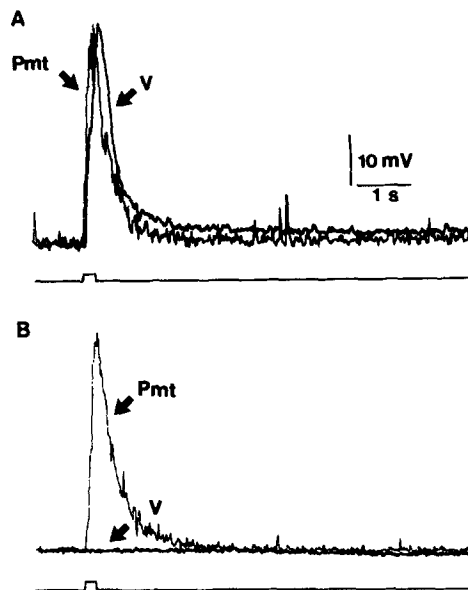


FIGURE 6. Use of aequorin to confirm calcium injections into light-sensitive (*A*) and -insensitive (*B*) regions of cells. The two cells in *A* and *B* were injected with aequorin, and the calcium-induced luminescence was monitored with a photomultiplier. The aequorin traces have been arbitrarily normalized. The peak luminescence was 120 counts per 10-ms bin in trace *A* and 320 counts per 10-ms bin in trace *B*. Injection of a pulse of 2 mM calcium in carrier solution that included 0.001% Triton X-100 produced a rapid transient rise in luminescence (PMT trace) that accompanied the calcium-induced depolarization. A similar injection of calcium into an insensitive region of another cell (*B*) produced a larger aequorin luminescence but no comparable depolarization. The bottom traces in *A* and *B* indicate the time of the pressure injections.

mediated, like adaptation of the light response, by a rise in intracellular free calcium. We therefore impaled cells with two blunt electrodes, one containing 100 μ M IP₃ in carrier solution, and the other containing 1 mM calcium aspartate in carrier solution.

Fig. 7 demonstrates that a 1–10-pl injection of calcium reversibly desensitized the photoreceptors' response both to a subsequent dim flash and to a 1–10-pl injection of IP₃. In this and three similar experiments, the injection of 1 mM calcium reversibly reduced the area under the IP₃ responses of four cells to $10 \pm 4\%$ and the area under light responses to $7 \pm 8\%$ of control values. After control injections of a much greater volume of carrier solution (estimated to be

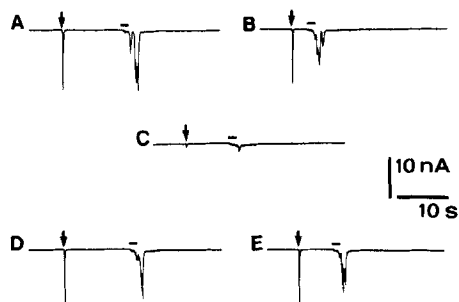


FIGURE 7. Reversible desensitization of responses to light flashes and IP_3 injection by calcium. The cell was impaled with two micropipettes and voltage-clamped. The voltage electrode contained 1 mM calcium aspartate in carrier solution; the current electrode contained $100 \mu M$ IP_3 in carrier solution. (A and B) Control responses to diffuse light flashes (duration, 10 ms; $\log_{10} [I] = -3$) at arrows and pressure injections of IP_3 (10 psi; 1.5 s) at bars. (C) Responses to same light flashes and IP_3 injections 30 s after pressure injection of 1–10 pl of solution from the pipette containing calcium. (D and E) Recovery of the responses to light and IP_3 3 min after the calcium injection.

10–100 pl) into four other cells, the area under the responses to IP_3 injections underwent a small, irreversible reduction to $78 \pm 23\%$ of control values, with a similar small, irreversible decline in the area under the light responses to $66 \pm 19\%$ of control values. Thus, injections of calcium, but not of carrier solution,

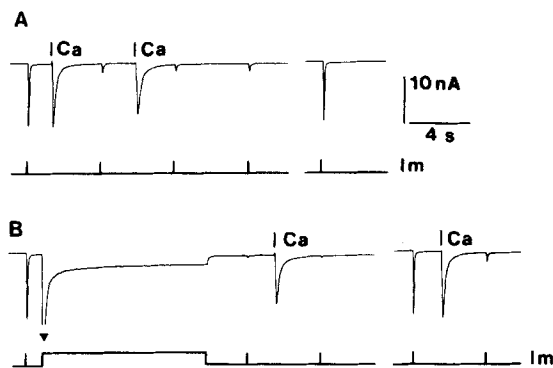


FIGURE 8. Adaptational properties of responses to calcium injections. Cells were voltage-clamped using one micropipette containing KCl and one containing 2 mM calcium aspartate in carrier solution. Diffuse light flashes (duration, 10 ms; $\log_{10} [I] = -3.5$) were delivered every 5 s, as indicated by the light monitor (lm) traces. (A) Pressure injection of calcium, indicated by the bars above the record (100 ms, 10 psi), elicited an inward current that desensitized the response to a subsequent light flash much more than the response to a subsequent injection of calcium. The 2-min break in the record allowed for the recovery of the light response. (B) Prolonged illumination of the same cell at the same intensity as the flashes desensitized the response to subsequent flashes much more than the response to pressure injection of calcium. The 3-min break in the record allowed for the recovery of the light response.

greatly and reversibly desensitized the photoreceptor's response to both light and IP_3 .

The calcium-activated current is only slightly adapted by light or by a prior injection of calcium. The inward current caused by a dim flash can be greatly suppressed (adapted) either by a prior injection of calcium (Figs. 1 and 7; Lisman and Brown, 1972) or by prior bright or prolonged illumination (Millecchia and Mauro, 1969). Similarly, the inward current produced by injections of IP_3 can also be adapted (Fig. 7; Fein et al., 1984; Brown et al., 1984). In this section, we show that the activation of inward current by calcium injection does not share these adaptation properties.

Photoreceptors were impaled in their most sensitive region with a pipette containing 2 mM calcium aspartate in carrier solution, and elsewhere with another pipette, containing 3 M KCl. Diffuse light flashes were delivered every 5 s. Fig. 8A shows the voltage-clamped response to two injections of calcium. The first injection activated the usual inward current and greatly desensitized the response to a subsequent light flash. Despite the large desensitization of the response to light, the response to a second calcium injection was only slightly diminished in amplitude and was of similar duration. The response to light was then allowed to recover (Fig. 8B) and a prolonged flash of the same intensity as the test flashes was delivered. The prolonged illumination adapted the receptor, greatly reducing the response to a subsequent light flash. However, an injection of calcium at this time activated a current that was only slightly smaller than (and had a duration similar to) the control response of Fig. 8A and the subsequent control obtained after recovery from light adaptation in Fig. 8B.

DISCUSSION

Characteristics of the Calcium-activated Current

Pressure injection of 1–10 pl of 1 or 2 mM calcium results in a transient inward current that depolarizes the photoreceptor. This inward current appears to be due to a rise in intracellular free calcium, since it can be abolished by the injection of a calcium buffer solution containing EGTA and its time course closely follows the rise in Ca_i caused by the injection, as indicated by aequorin luminescence. The rectification, reversal potential, and permeability to sodium and lithium of the calcium-activated current are similar to those of the light-activated current, which strongly suggests that both currents arise from the activation of the same ionic channels. In support of this assertion, we found that only the R-lobe, which contains structures specialized for transduction, is sensitive to calcium injections. However, definitive proof of this identity must await confirmation by the patch-clamp technique, recently applied to ventral photoreceptors by Bacigalupo and Lisman (1983). It remains to be determined whether calcium directly activates channels or whether it initiates the release of another messenger that opens the channels.

Studies on the voltage-activated currents of ventral photoreceptors have described a reduction in a voltage-activated potassium current after prolonged ionophoretic injection of calcium (Pepose and Lisman, 1978; Chinn and Lisman, 1984). This effect is thought to mediate the ability of sustained, intense illumi-

nation to slowly depress the voltage-activated outward current (Lisman and Brown, 1971; Leonard and Lisman, 1981). Since the voltage-activated potassium conductance is negligible at potentials close to rest, we would not expect the inward currents induced by pressure injection of calcium in this article to be contaminated at the resting potential by modulation of voltage-activated conductances. Contamination might be expected when the cell is depolarized and the potassium conductance is activated. However, our experiments on the reversal of the calcium-activated conductance appear to show a unique reversal potential of +10 to +20 mV. We have no evidence for a second, slower component caused by a potassium conductance that fails to reverse at these potentials. It is possible that, like brief flashes (Lisman and Brown, 1971), brief pulses of calcium fail to depress the voltage-activated conductance or that the density of the voltage-activated channels in the R-lobe is much less than the calcium-activated channels.

Sensitivity of Excitation and Adaptation to Calcium

Brown and Lisman (1975) did not observe inward currents during ionophoretic injection of calcium. Their ionophoretic injections, of ~60 s duration, induced a desensitization of the response to light flashes similar to that induced by our 30–200-ms pressure injections. We suggest that the difference in the excitatory effect arises from the rapidity of the delivery of calcium by pressure injection, which causes a large initial transient rise in Ca_i lasting a few seconds (see Fig. 6). We therefore propose that the concentration of Ca_i required in the body of the R-lobe to induce an inward current is much higher than that required to desensitize the photoreceptor. This proposal would explain why desensitization persists long after the calcium-activated depolarization and inward current have disappeared (Figs. 1 and 5). Since desensitization of the photoreceptor during steady illumination is associated with micromolar changes in Ca_i (Levy and Fein, 1985), we tentatively suggest that excitation of the photoreceptor by calcium requires greater than micromolar changes of Ca_i in the bulk of the cytosol.

There are two possible reasons for the lower sensitivity to injected calcium of excitation compared with that of adaptation. The binding sites for calcium that mediate excitation may have a lower affinity for calcium, or they may be placed in a calcium-buffered compartment that is more remote from the site of injection than the compartment containing the binding sites that mediate adaptation. The small space between the base of the microvilli and the subrhabdomic cisternae of the endoplasmic reticulum (Calman and Chamberlain, 1982), which sequesters calcium (Walz and Fein, 1983), may comprise such a compartment. Unfortunately, uncertainties in the speed of dilution of the injected bolus of calcium and the speed of intracellular calcium buffering make it impossible to estimate the calcium concentration at the plasma membrane after injection. Both factors might reasonably be expected to greatly reduce the calcium concentration at the membrane, compared with the 1 or 2 mM calcium that is injected.

Are Excitation and Adaptation by Light or IP_3 Mediated by Calcium Release?

Insofar as pressure injection of calcium appears to activate the light-sensitive conductance and desensitize the response to subsequent light flashes or IP_3

injections (Brown et al., 1984; Fein et al., 1984), it mimics excitation and adaptation of the photoreceptor by both of these agents. Both light and IP_3 cause a rise in intracellular calcium (Brown and Rubin, 1984; Corson et al., 1984). Estimates of the peak transient increase in Ca_i after a bright flash have been given by Brown et al. (1977) and Levy and Fein (1985). The estimate of $100 \mu M$ given by Brown et al. (1977) should be regarded as a lower limit because of the localization of the arsenazo signal (Harary and Brown, 1984). The additional problem of the slow response times of the calcium electrodes used by Levy and Fein (1985) makes their estimate of $40 \mu M$ a lower limit as well.

Both pressure injection and ionophoretic injection of calcium desensitize the response to subsequent light flashes (Lisman and Brown, 1972). Stern et al. (1982) showed that illumination of the R-lobe is much more effective in initiating adaptation than illumination of the A-lobe. Our finding that calcium is also more effective in desensitizing the receptor when injected into the R-lobe, as opposed to the A-lobe, adds further evidence for the hypothesis that light adaptation is mediated by a rise in Ca_i (Lisman and Brown, 1972) and is compatible with the slow and limited diffusion of calcium within the cell (Harary and Brown, 1984; Levy and Fein, 1985).

Several differences exist, however, between excitation by calcium and by light or IP_3 . First, excitation by calcium resulted in a smoothly graded inward current that rose during the period of the injection, rather than the discrete waves of current evoked by light (Yeandle and Spiegler, 1973) or the waves and bursts of current that could continue for many seconds after an injection of IP_3 (Fein et al., 1984). This difference might be expected if IP_3 or photons released calcium in bursts very close to the binding sites for calcium.

A second difference between the responses to light, IP_3 , and calcium is that the response to calcium injection was much less desensitized by a prior injection of calcium than was the response to light or IP_3 . The pathway of excitation activated by calcium appears to lack the calcium-activated sites that adapt the receptor. This difference, however, does not necessarily mean that excitation by light or IP_3 is mediated through a pathway other than the release of calcium, since it is possible that calcium exerts its effects through inhibition of calcium release by light or IP_3 . This is clearly a testable prediction.

The most important difference between the responses to light and calcium is that injection of a calcium buffer containing EGTA abolished the response to calcium injection but only slowed and diminished the dark-adapted response to light. The slowing and diminution of the dark-adapted light response by EGTA may indicate a role for calcium release in the excitation of the dark-adapted photoreceptor by light, although the possibility that EGTA acts via means other than the buffering of calcium cannot be ruled out. It is not clear whether calcium is a sufficient or necessary transmitter of the visual cascade. It may be that calcium release is the sole mediator of excitation by light and that EGTA slows, but does not abolish, the light response because light releases calcium into some confined compartment in the cell, saturating the available EGTA. We stress that there is, at present, no direct experimental evidence to suggest that calcium release by light is sufficient to mediate excitation. Alternatively, and more likely, calcium release by light may be a parallel pathway of excitation that accelerates

the release of another, unknown transmitter, but is not necessary for excitation (Payne and Fein, 1986).

We show in the following article (Payne et al., 1986) that injection of EGTA greatly reduces the response to IP₃ injection and we therefore think it likely that excitation and adaptation by IP₃ are mediated by a rise in intracellular calcium.

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