Excitation and Adaptation of *Limulus* Ventral Photoreceptors by Inositol 1,4,5 Trisphosphate Result from a Rise in Intracellular Calcium

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ABSTRACT   Single pressure injections of 1–10 pl of inositol 1,4,5 trisphosphate (IP_3) or inositol 4,5 bisphosphate [$I(4,5)P_2$] excite *Limulus* ventral photoreceptors by inducing rapid bursts of inward current. After excitation by IP_3, responses to subsequent injections of IP_3 or light flashes are often reversibly diminished (adapted). Single injections of IP_3 and $I(4,5)P_2$ are effective at concentrations in the injecting pipette of 20 μM to 1 mM. Single injections of inositol 1,4 bisphosphate are ineffective at concentrations of 100–500 μM. Excitation by IP_3 or $I(4,5)P_2$ is accompanied by a rise in intracellular free calcium, as indicated by aequorin luminescence. Prior injection of calcium buffer solutions containing 100 mM EGTA greatly diminishes the total charge transferred across the plasma membrane during excitation by IP_3 or $I(4,5)P_2$, which suggests that a rise in Ca_i is necessary for excitation by the inositol polyphosphates. Adaptation of the response to light by IP_3 is also abolished by prior injection of EGTA. In the same cells, the response to brief light flashes is slowed and diminished in amplitude by the injection of calcium buffer, but the charge transferred during the response is not significantly diminished. This suggests that light has access to a pathway of excitation in the presence of EGTA that is not accessible to intracellularly injected IP_3.

INTRODUCTION

Inositol 1,4,5 trisphosphate (IP_3) and related compounds both excite and adapt *Limulus* ventral photoreceptors (Brown et al., 1984; Fein et al., 1984). IP_3 is the water-soluble product of the hydrolysis of phosphatidylinositol 4,5 bisphosphate ($PtdInsP_2$), a minor component of plasma membrane phospholipid (Grado and Ballou, 1961; Tomlinson and Ballou, 1961). Hydrolysis of inositol lipids was implicated by Michell (1975) in the mobilization of calcium in response to hormonal stimulation. Berridge (1983) proposed that the IP_3 released by hy-

drolysis of $PtdInsP_2$ acted as a second messenger that released calcium from internal stores. Streb et al. (1983), Irvine et al. (1984), and others (for review, see Berridge and Irvine, 1984) have confirmed that IP_3 and inositol 4,5 bisphosphate [$I(4,5)P_2$] release calcium from a nonmitochondrial store in permeabilized cells, possibly the endoplasmic reticulum (Streb et al., 1984). Inositol 1,4 bisphosphate [$I(1,4)P_2$] is much less effective than IP_3 or $I(4,5)P_2$ in releasing calcium from permeabilized cells (for review, see Berridge and Irvine, 1984).

The inositol polyphosphates that release calcium from permeabilized cells are also those able to excite and adapt ventral photoreceptors (Brown et al., 1984; Fein et al., 1984). We therefore decided to investigate the extent to which excitation and adaptation by these compounds are mediated by their ability to release calcium. We have shown in the preceding article (Payne et al., 1986) that calcium both excites and adapts ventral photoreceptors when pressure-injected into the region of the cell that is most sensitive to light. A rise in calcium therefore may be sufficient for the actions of IP_3. In this article, we demonstrate that IP_3 causes a rise in intracellular calcium and that this rise is necessary for excitation and adaptation of the photoreceptor by IP_3.

The results of this article have previously been published in abstract form (Corson et al., 1984; Payne et al., 1984).

METHODS

Recording and Stimulation

The methods of stimulation, recording, and pressure injection are described in the preceding article (Payne et al., 1986).

Chemicals and Injection Solutions

The sources of EGTA, MOPS, and HEPES are described in the preceding article (Payne et al., 1986). Quin 2 (Tsien, 1980) was obtained from Sigma Chemical Co., St. Louis, MO. IP_3, $I(1,4)P_2$, and $I(4,5)P_2$ were prepared according to the methods of Irvine et al. (1984). The carrier solution, containing 100 mM potassium aspartate and 10 mM HEPES, pH 7, was used to dilute solutions injected into the cell.

The free calcium concentration in the calcium buffer solutions containing EGTA was measured with a calcium-sensitive macroelectrode similar to that used by Levy and Fein (1985). The methods of preparation and injection of aequorin were as described by Payne et al. (1986). Luminescence from the aequorin was collected with a long-working-length 40× objective (UD 40/.65C, Carl Zeiss, Inc., Thornwood, NY) and monitored with a photomultiplier (model 9558A, Thorn-EMI, Plainview, NY) operated in analog mode at 1,200 V.

Artificial Seawaters

The compositions of the artificial seawaters (ASWs) used in the experiments of this paper are given in Table I of the preceding article (Payne et al., 1986). All salines had a pH of 7.

RESULTS

Excitation of Ventral Photoreceptors by IP_3 and $I(4,5)P_2$

Our observations on the excitation of ventral photoreceptors by injection of inositol polyphosphates accord with those reported by Fein et al. (1984).

Single injections of 1–10 pl of 100 μM IP$_3$ typically induced a series of bursts of inward current, up to 25 nA in amplitude, each lasting ~1 s (for an example, see Fig. 1). The bursts of inward current after an injection of IP$_3$ could continue for many seconds. The bursts usually had an irregular waveform (see Fig. 1). Bursts of inward current similar to those of Fig. 1 could also be evoked by injection of 100 μM I(4,5)P$_2$. Single injections of 1–10 pl of 20 μM IP$_3$ or I(4,5)P$_2$ were also effective in exciting the photoreceptor. 2 μM IP$_3$ or I(4,5)P$_2$ excited photoreceptors after multiple injections. We observed no excitation after single injections of up to 500 μM I(1,4)P$_2$, although multiple injections did cause excitation. Thus, I(1,4)P$_2$ was much less effective in exciting cells than IP$_3$ or I(4,5)P$_2$.
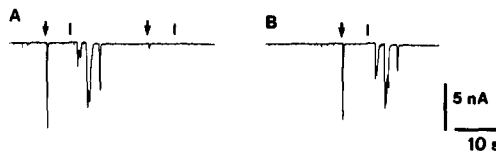

FIGURE 1.   IP$_3$ injection desensitizes responses to subsequent IP$_3$ injections and to subsequent light flashes. The cell was impaled with one micropipette containing 3 M KCl and one containing 100 μM IP$_3$ in carrier solution. The latter was used to pass current in order to clamp the cell at its resting potential (−55 mV). (A) Responses to 1–10-pl injections of 100 μM IP$_3$ in carrier solution (bars) and 10-ms light flashes (arrows). The responses to the second light flash and to a second injection of IP$_3$ were greatly attenuated. (B) Recovery of the responses to light and IP$_3$ 5 min after the responses in A. The intensity of the flash (attenuation, 3.5 log$_{10}$ units) was chosen so as not to significantly desensitize the cell to injections of IP$_3$ or flashes delivered 5 s later. Injection pressure, 15 psi; injection duration, 500 ms.

Fein et al. (1984) and Brown et al. (1984) have demonstrated that the reversal potential of the inward current produced by an injection of IP$_3$ is the same as that of the light response, which suggests that the same channels are opened by the two agents. Light-activated channels in ventral photoreceptors have been reported as having conductances of the order of 10–100 pS (Wong, 1978; Bacigalupo and Lisman, 1983). If the channels opened by light and IP$_3$ are identical, then each burst of inward current must arise from the simultaneous opening of tens of thousands of channels.

IP$_3$ Densensitizes Subsequent Responses to Light Flashes or to Injections of IP$_3$

Single injections of 100 μM IP$_3$ desensitized subsequent responses both to diffuse light flashes (Brown et al., 1984; Fein et al., 1984) and to further injections of IP$_3$ (Fig. 1A). The recovery of the responses to light and IP$_3$ was typically complete within 5 min (Fig. 1B).

The degree of desensitization of the response to light was variable from cell to cell, but reliable from injection to injection into the same cell. Out of a total of 34 cells that were excited by single injections of 100 μM IP$_3$, 19 cells displayed reversible desensitization of their responses to subsequent diffuse light flashes, and 7 displayed facilitation of the light responses (Fein et al., 1984); in 8 of the cells, there was no effect of the injection on the light response. A possible

explanation for the variable effects of the injections is the localization of the induced desensitization within the cell. Fein et al. (1984) obtained more reliable desensitizations of responses to flashes that were confined to the area of the injection, rather than diffused, as the flashes used in this article were. The injection of nine cells with 2 μM IP$_3$ induced no reversible desensitization, even after multiple injections.

I(4,5)P$_2$ could also desensitize the cell's response to diffuse light. As with IP$_3$, the degree of desensitization was highly variable. 4 out of 11 cells injected with 100 μM I(4,5)P$_2$ were reversibly desensitized. Injections of 100 and 500 μM I(1,4)P$_2$ (six cells and three cells, respectively) failed to reversibly desensitize the response of any of the receptors to diffuse light. Thus, I(1,4)P$_2$ was less effective in desensitizing the cell's response to light than IP$_3$ or I(4,5)P$_2$.

Elevation of Intracellular Free Calcium by IP$_3$

To find out whether IP$_3$ is able to elevate Ca$_i$ in ventral photoreceptors, we attempted to detect IP$_3$-induced changes in Ca$_i$ with the calcium-sensitive, luminescent protein aequorin (Shimomura et al., 1962). Aequorin has been previously used to detect light-induced changes in Ca$_i$ in these cells (Brown and Blinks, 1974). Measurements of aequorin luminescence were made in cells bathed in ASW containing 1 mM calcium (normally 10 mM) to increase the sensitivity of the cell (Millecchia and Mauro, 1969). Cells were impaled with two pipettes containing 1 mM IP$_3$ in carrier solution and 7 mg/ml aequorin, respectively. Aequorin was injected into the cell before injection of IP$_3$. Fig. 2, A and B, shows that injection of 1 mM IP$_3$ depolarized the photoreceptor and also elevated Ca$_i$. The depolarization usually consisted of a single burst showing multiple peaks.

We showed in Fig. 1 that a 1–10-pl injection of IP$_3$ caused a reversible decrease in the response to a subsequent IP$_3$ injection. Fig. 2, C–E, shows that when this occurs, the concomitant rise in Ca$_i$ is greatly diminished. A control injection of IP$_3$ elicited a depolarization and an associated rise in Ca$_i$ (Fig. 2C). A second injection 30 s later (Fig. 2D) elicited a smaller (adapted) depolarization and a much smaller rise in Ca$_i$ (Fig. 2D). Responses to dim test flashes at this time (not shown) were also depressed in amplitude. 3 min later, the test flash responses had recovered in amplitude and a third injection of IP$_3$ elicited a depolarization and aequorin luminescence (Fig. 2E) comparable to those elicited by the initial injection. The diminution of the aequorin luminescence associated with the second injection also served as a control against the possibility that the increase in luminescence was due to contamination of the injection solution by calcium.

Although we were able to detect an IP$_3$-induced rise in Ca$_i$, not every IP$_3$-induced depolarization (e.g., Fig. 2D) was associated with detectable aequorin luminescence. It is possible that our measuring system was not sensitive enough to detect small elevations in Ca$_i$. We note that aequorin measurements similar to ours (Brown and Blinks, 1974) have proven insufficiently sensitive to detect the small elevations in Ca$_i$ ($<$5 μM) that have been detected with calcium electrodes during sustained illumination (Levy and Fein, 1985).

We compared the time course of the aequorin luminescence with the time course of its associated IP$_3$-induced depolarization to see whether the rise in Ca$_i$

might precede the depolarization. We found that the peak of the aequorin luminescence (mean ± SD of time to peak, t_p = 578 ± 112 ms) preceded the peak of the IP$_3$-induced depolarization (t_p = 620 ± 112 ms) in 18 measurements, but lagged behind it in 8 of 26 measurements in nine cells. For these cells, the rising edge of the aequorin luminescence can lead (Fig. 2A) as well as lag (Fig. 2B) the rising edge of the depolarization when the waveforms of both are
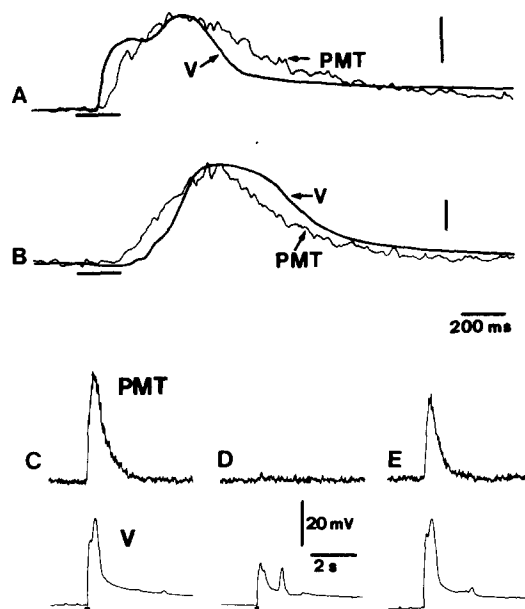

FIGURE 2. Aequorin luminescence and membrane depolarization elicited by IP$_3$ injection into a ventral photoreceptor. (A and B) Injection of 1 mM IP$_3$ in carrier solution (bars) into two different cells elicited both depolarization of the cell membrane (traces labeled V) and aequorin luminescence (traces labeled PMT), which indicated a rise in Ca$_i$. The bars to the right of A and B indicate a scale of 20 mV for the voltage traces. The PMT trace is arbitrarily normalized to the peak of the voltage trace. (C–E). Injections of 1 mM IP$_3$ into the same cell as in A. A control injection of IP$_3$ (C) was followed by a second injection 30 s later (D). The second injection elicited a smaller (adapted) depolarization. The corresponding PMT record of D indicates that the Ca$_i$ rise monitored by aequorin was much smaller. A third injection 3 min later elicited a depolarization and aequorin luminescence (E) comparable to those elicited by the initial injection.

arbitrarily normalized to equal amplitudes. We were therefore unable to ascertain whether the rise in Ca$_i$ precedes or follows the IP$_3$-induced depolarization. These findings indicate that IP$_3$ produces a rise in Ca$_i$ that can be adapted and that appears to be fast enough to participate in adaptation and perhaps excitation.

In a series of experiments on eight cells, we found that 1–10-pl injections of 1 mM I(4,5)P$_2$ share the ability of injections of IP$_3$ to cause an increase in aequorin luminescence when injected into the photoreceptor.

Effects of Calcium Buffers on the Response to Light and to IP₃

Control injections. The remaining experiments of this article required impalement of the photoreceptor with two micropipettes. One electrode, placed in the most sensitive region of the cell, contained 100 μM IP$_3$ in carrier solution. The other, placed in a different region of the cell, contained a solution whose effects on the response of the cell to IP$_3$ we wished to investigate. The complete procedure of a control experiment is shown in Fig. 3. In this experiment, the
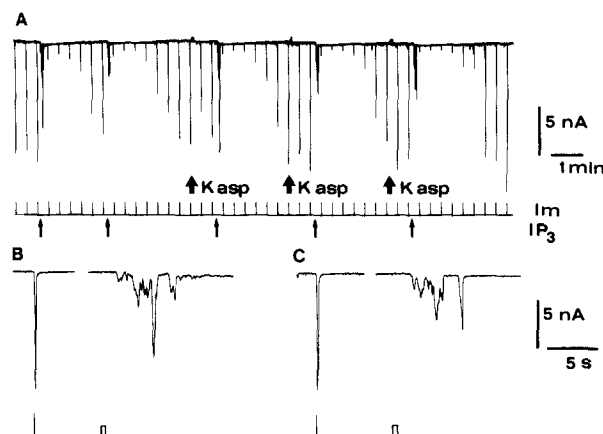

FIGURE 3. Control experiment showing that injections of carrier solution do not modify excitation and adaptation of the cell by IP$_3$. The cell was impaled with one micropipette containing carrier solution and one containing 100 μM IP$_3$ in carrier solution. The latter was used to pass current in order to clamp the cell at its resting potential. (*A*) Complete record of the voltage-clamp current throughout the experiment (upper trace). The light monitor trace (lm) indicates the times of 10-ms flashes attenuated by 3.5 log$_{10}$ units. At the thin arrows, 1–10 pl of 100 μM IP$_3$ (500 ms, 15 psi) was injected to excite and adapt the cell. At the thick arrows, labeled K asp, a series of five 200-ms, 15-psi pressure pulses was applied to the other pipette, each series ejecting 5–50 pl of carrier solution. (*B*) Currents elicited by the first control injection of IP$_3$ (right-hand trace) and by the preceding light flash (left-hand trace). The marks below traces show the times of the flash or the injection. (*C*) Currents elicited by the final injection of IP$_3$ and by the preceding light flash after three sets of injections of carrier solution.

second pipette contained carrier solution. The receptor was voltage-clamped to its resting potential, using the pipette containing IP$_3$ to pass current, while recording potential through the other pipette. Dim, brief flashes (lm trace) were delivered every 30 s. At the times indicated by the thin arrows at the bottom of Fig. 3*A*, a single bolus of 1–10 pl of 100 μM IP$_3$ was injected. The current trace shows excitation of the receptor by the injection, followed by reversible desensitization of the light response. Fig. 3*B* illustrates the first of these control responses to light and IP$_3$ on a faster time scale.

Two control injections of IP$_3$ were delivered to the cell of Fig. 3*A*, with sufficient time between to allow for the recovery of the light response. Then, at

the times indicated by the thick arrows above the lm trace, 5–50 pl of carrier solution was injected by a rapid series of five injections. The injections of carrier solution were repeated three times. Fig. 3A demonstrates that the injections of carrier solution had little or no effect on excitation or desensitization due to IP$_3$ or excitation by light. Fig. 3C illustrates responses to light and IP$_3$ after the final injection of carrier solution. In a total of four cells, 10–100-pl injections of
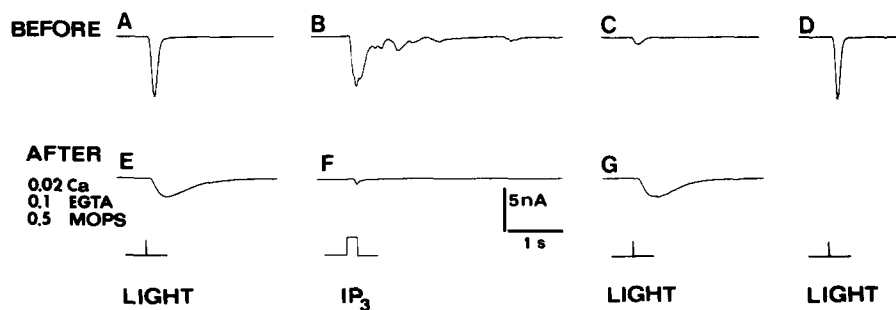

FIGURE 4. Effect of the injection of a calcium buffer solution containing calcium and EGTA, in the ratio 0.2 Ca/EGTA, on IP$_3$-induced excitation and adaptation. The cell was impaled with one electrode containing 100 μM IP$_3$ in carrier solution and another electrode containing 100 mM EGTA, 20 mM Ca(OH)$_2$, and 500 mM MOPS, neutralized with KOH and having a free calcium concentration of 10^{-7} M. The ratio of 0.2 Ca/EGTA was chosen to maximize the area under the light response after injection and to accentuate the differing effects of calcium buffer on the light and IP$_3$ responses. Either solution could be pulse-pressure–injected into the cell. The same flash intensity ($\log_{10} [I = -4$) and duration (10 ms) were used throughout the experiment. The intensity was chosen so as not to appreciably adapt the response to an IP$_3$ injection occurring 10 s after the flash. (A–D) Recordings before Ca/EGTA/MOPS injection. (A) Control light response. (B) Response to 100 μM IP$_3$, injected 10 s after the flash in A by a 200-ms, 20-psi pressure pulse. (C) Desensitized light response recorded 10 s after the IP$_3$ injection in B. (D) Recovery of the light response 110 s after the IP$_3$ injection in B. (E–G) Recordings made after 10–100-pl injection of Ca/EGTA/MOPS, delivered by 10 brief (200 ms, 20 psi) pressure pulses. (E) Response to a light flash 50 s after Ca/EGTA/MOPS injection. (F) Responses to 100 μM IP$_3$ injection (200 ms, 20 psi) recorded 10 s after the flash in E. (G) Response to a light flash recorded 10 s after the IP$_3$ response in F. The markers below the traces show the times of flashes or IP$_3$ injections. The concentrations quoted in the figure are molar.

carrier solution, delivered in two sets of five brief injections (as in Fig. 3), only slightly reduced the area under the response to IP$_3$ to 78 ± 28% (mean ± SD) of the control values and similarly slightly reduced the area under the light response to 66 ± 19% of the control values.

Effects of injection of solutions containing EGTA. To find out whether a rise in intracellular free calcium was necessary for the actions of IP$_3$, we injected cells with a calcium buffer solution containing EGTA before injecting it with IP$_3$. The experimental protocol was similar to that of the control experiment de-

scribed in the preceding section. One electrode contained 100 μM IP_3 in carrier solution, and the other contained 0.02 M $Ca(OH)_2$, 0.1 M K_2EGTA, and 500 mM MOPS, neutralized with KOH. The calcium buffer solution had a measured free calcium concentration of 10^{-7} M.

Excitation and adaptation of the photoreceptor by IP_3 are demonstrated in Fig. 4, A–D. Fig. 4A shows the dark-adapted response to a light flash, followed by an injection of IP_3 (Fig. 4B). 10 s after the injection of IP_3, the photoreceptor
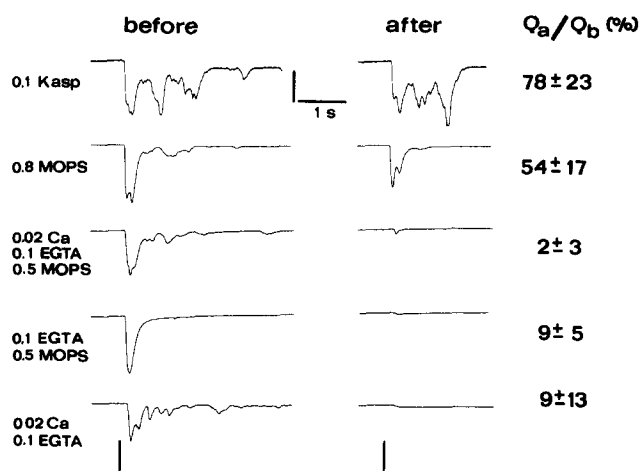

FIGURE 5.   Effects of the injection of 10–100 pl of the test solutions listed in the left-hand column on the response of ventral photoreceptors to 1–10-pl injections of 100 μM IP_3. The total charge transferred across the cell membrane during the IP_3 response that followed the injection of the test solutions is expressed in the right-hand column as a percentage of the charge transferred during previous control injections of IP_3 (Q_a/Q_b; mean ± SD of several experiments on different cells). The traces show voltage-clamp currents caused by 1–10-pl injections of IP_3 recorded before (left) and after (right) the injection of test solutions. The responses before injection are scaled to similar amplitudes in the figure. The vertical bar represents 2 nA in A, 8 nA in B, and 4 nA in C, D, and E. The numbers of cells used to collect the ratios of charge transferred are 4 (A), 6 (B), 5 (C), 5 (D), and 2 (E). The concentrations quoted in the figure are molar.

was desensitized to light (Fig. 4C) and recovered 2 min later (Fig. 4D). After injection of 10–100 pl of EGTA, the response to light was slowed and diminished in amplitude (Fig. 4E; Lisman and Brown, 1975). The response to IP_3 was greatly diminished in amplitude (Fig. 4F) after the injection of EGTA, and a light response elicited 10 s after the injection of IP_3 had the same amplitude as that elicited before (Fig. 4, E and G). The calcium buffer solution therefore greatly reduced excitation and desensitization of the photoreceptor by IP_3 and it slowed and diminished the amplitude of the dark-adapted response to a light flash.

We varied the composition of the solution injected before the injection of IP_3. Figs. 5 and 6 illustrate responses to IP_3 and light, respectively, before and after the injection of 10–100 pl of solutions containing the substances named at the

left of the figures. Three calcium buffer solutions were used: 0.1 M EGTA alone, 0.1 M EGTA with 0.02 M $Ca(OH)_2$, and 0.1 M EGTA with 0.02 M $Ca(OH)_2$ and 0.5 M MOPS. Control injections having low ionic strength (carrier solution) and high ionic strength (0.8 M MOPS) were also included. The average areas under the responses after injection of these solutions (the total charge transferred across the cell's membrane during the response) are tabulated on the right of the figures, expressed as a percentage of their values before injection. Control injections of stock solution and 0.8 M KMOPS (pH 7) slightly reduced the areas under the responses to both light and IP_3. The solutions containing EGTA
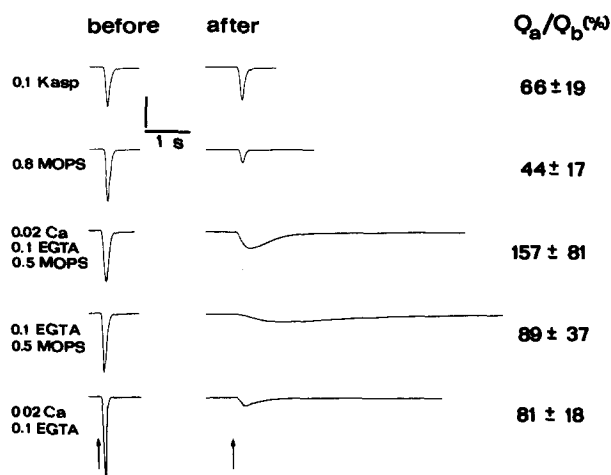

FIGURE 6. Effects of the injection of 10–100 pl of the test solutions listed in the left-hand column on the response of ventral photoreceptors to a 10-ms flash. The total charge transferred across the cell membrane during the light response that followed the injection of the test solutions is expressed in the right-hand column as a percentage of the charge transferred during previous control flashes (Q_a/Q_b; mean ± SD of several experiments on different cells). The traces show voltage-clamp currents caused by 10-ms diffuse flashes, attenuated 3.5 $\log_{10}$ units, recorded before (left) and after (right) the injection of test solutions. The responses before injection are scaled to similar amplitudes in the figure. The vertical bar represents 2 nA in A, 8 nA in B, and 4 nA in C, D, and E. The numbers of cells used to collect the ratios of charge transferred are 4 (A), 6 (B), 5 (C), 5 (D), and 2 (E). The concentrations quoted in the figure are molar.

reduced the areas under the responses to IP_3 to a much greater extent than did the control injections. In addition, the solutions containing EGTA alone, or Ca and EGTA in a ratio of 0.2, greatly slowed and diminished the amplitude of the light responses. In contrast to the IP_3 responses, however, the areas under the responses to light were not significantly reduced after injection of solutions containing EGTA. Thus, injections of EGTA selectively reduce the charge transferred during the response to injections of IP_3 but not during the response to light.

None of the effects of EGTA reported in this article were reversible within 20 min of injection, although the cells' resting potential and the amplitude of the
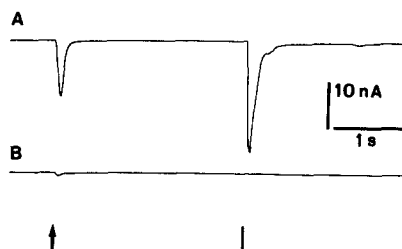
FIGURE 7. Effect of injection of 10–100 pl of a calcium buffer solution containing 0.08 M Ca(OH)$_2$, 0.1 M EGTA, and 0.5 M MOPS, neutralized with KOH on the response of a ventral photoreceptor to a 10-ms flash of diffuse light (arrow) and to a 1-10-pl injection of 100 μM IP$_3$ in carrier solution (bar). Responses are shown before (upper trace) and 1 min after the injection of the calicum buffer solution. Log$_{10}$ flash attenuation = 4; injection duration, 100 ms; pressure, 20 psi.

light response remained stable. Similar experiments using 100 μM I(4,5)P$_2$ instead of IP$_3$ resulted in similar reductions in the adaptation and excitation resulting from the injection of I(4,5)P$_2$ into a total of 11 cells.

To investigate the effects of increased calcium concentration in the buffer solution, we also injected cells with 10–100 pl of a solution containing 0.08 M Ca(OH)$_2$, 0.1 M EGTA, and 0.5 M MOPS, neutralized with KOH. This calcium buffer solution had a free calcium concentration of 10^{-6} M. The effect of this injection on the response to light and IP$_3$ is shown in Fig. 7. As with the buffer solutions containing Ca and EGTA in a ratio of 0.2, the response to 100 μM IP$_3$ was greatly diminished after injection of 0.8 Ca/EGTA. The response to light was also diminished in area, but to a lesser extent than that under the response to IP$_3$. After injection of 0.8 Ca/EGTA, the response to light was also much faster than after the injection of 0.2 Ca/EGTA (Payne and Fein, 1984). Thus,
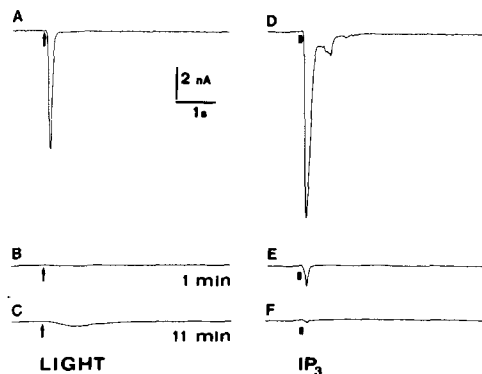

FIGURE 8. Effect of injection of 5–20 pl of a solution containing 0.1 M Quin 2 and 0.01 M HEPES, neutralized with KOH on the response of a ventral photoreceptor to 10-ms flashes of diffuse light (A–C) and to 1-10-pl injections of 100 μM IP$_3$ in carrier solution (D–F). Responses are shown before (A and D) and 1 (B and E) and 11 (C and F) min after the injection of Quin 2. Log$_{10}$ flash attenuation = 4, delivered at the arrows; injection duration, 100 ms; pressure, 20 psi, delivered at the bars.

increasing the calcium concentration in the calcium buffer reduced the amplitude and time scale of the light response after injection, but the total charge transferred during the light response was still greater after injection than that transferred during the response to IP_3.

Quin 2 abolishes the response to IP_3. We decided to compare the effects of other chelators of calcium on the response to injections of 100 μM IP_3. The calcium chelator and indicator Quin 2 (Tsien, 1980) has a pK for calcium binding of 7, a high selectivity for calcium over magnesium, and negligible binding of protons. Fig. 8 illustrates responses to light and IP_3 recorded before and after injection of 5–50 pl of 0.1 M Quin 2. No calcium was added to the Quin 2, so we would expect the free calcium concentration to be $<10^{-7}$ M. 1 min after the injection, the responses to both IP_3 and light were greatly reduced. The response to light recovered somewhat over the next 10 min. The area under the light response recovered to 55% of its control value. The response to IP_3 did not recover. Thus, 10 min after the injection of Quin 2, the effects of the injection were qualitatively similar to those of EGTA. Similar effects of injections of Quin 2 on the responses to light and IP_3 were observed in a total of three cells. Similar effects of Quin 2 on the light response alone were observed in two other cells that were not injected with IP_3.

As regards the initial abolition of the response to light, we note that Quin 2 may chelate far more of the cell's calcium on first entering the cell than would an injection of the weaker buffer EGTA, thereby reducing the intracellular calcium concentration to a greater extent before it is restored by leakage of calcium into the cell. Bolsover and Brown (1985) showed that the depletion of calcium within the cell can lead to a collapse of the light response.

DISCUSSION

IP_3 excites and adapts ventral photoreceptors. Excitation and adaptation appear to have similar dependences on the concentration of IP_3 in the pipette, both being induced by concentrations as low as 20 μM (Fein et al., 1984). $I(4,5)P_2$ shares this ability to excite and adapt the photoreceptor, but $I(1,4)P_2$ is at least 10 times less potent. Both of the active inositol polyphosphates cause a rapid rise in Ca_i, as indicated by aequorin luminescence. In a recent paper, Brown and Rubin (1984) also described the ability of IP_3 to raise Ca_i in ventral photoreceptors and showed that lowering the extracellular calcium by introducing EGTA into the bathing solution leads to an increased, rather than a decreased, aequorin luminescence after injection of IP_3. Their experiment, which we have confirmed, suggests that the calcium is released from internal stores rather than entering the cell from the bathing medium. All these findings are consistent with the hypothesis (Berridge, 1983) that IP_3 releases calcium from inside cells. The work in this article shows that there is a rapid IP_3-induced rise in Ca_i in an intact, living cell.

The effects of an injection of IP_3 or $I(4,5)P_2$ can be greatly diminished by prior injection of solutions containing calcium buffers. Rubin and Brown (1985) have also shown that the calcium chelators EGTA and BAPTA (Tsien, 1980) greatly reduce the response of ventral photoreceptors to IP_3. It is possible that these compounds abolish the response to IP_3 by some means other than that

related to their ability to buffer calcium. However, we note that the cells' resting potential and the total charge transferred during the light response are not affected by the injection of calcium buffers. Both of these might be expected to decline if the calcium buffers challenged the viability of the cells. The assertion that the calcium buffers act by abolishing a necessary rise in Ca_i is supported by the results of the preceding article (Payne et al., 1986), in which we show that pressure injection of calcium into the light-sensitive R-lobe of the photoreceptor also excites and adapts it. The rise in Ca_i caused by IP_3 may therefore be both necessary and sufficient for its actions.

To prove rigorously that IP_3 acts solely by causing a rise in Ca_i, we would have to demonstrate that the IP_3-induced rise in Ca_i is sufficiently large to account for excitation. We have shown that, in most cells, the time to peak of the aequorin luminescence does not lag that of the depolarization induced by IP_3. However, we have so far been unable to demonstrate clearly that the rising edge of the luminescence always precedes the depolarization. Nor have we been able to calibrate our aequorin signals to determine whether the rise in Ca_i is of sufficient magnitude to induce excitation. To solve these problems, we need to know a great deal more about the sensitivity of our measuring system, the localization of the rise in Ca_i within the cell, and the access that aequorin has to the site of the rise of Ca_i. Using a photon-counting photomultiplier to improve sensitivity, we have recently obtained evidence of bursts of calcium release accompanying bursts of depolarization induced by 100 μM IP_3 (Corson, D. W., and A. Fein, manuscript in preparation).

Is Excitation by Light Mediated by a Release of IP₃?

Our evidence that IP_3 and $I(4,5)P_2$ may act via a rise in Ca_i has several implications for the hypothesis that visual excitation in ventral photoreceptors is mediated by the light-induced release of IP_3 or a closely related compound (Fein et al., 1984; Brown et al., 1984).

The response to light is slowed and diminished in amplitude by the injection of calcium buffers (Lisman and Brown, 1975), but the total charge transferred during the response is essentially unaffected. In the same cells, however, the charge transferred during the response to IP_3 is greatly diminished. In the presence of EGTA, it appears that light has access to a pathway for opening channels that is not accessible to exogenously applied IP_3. One possible explanation for this result is that, in addition to releasing IP_3, light releases some other, as yet unknown transmitter that opens channels without acting via a rise in Ca_i. If this is the case, then the slowing of the response to light by EGTA and Quin 2 might indicate that calcium release by light accelerates the reactions of the pathway that is not mediated by calcium release (Payne and Fein, 1986). Stieve and Bruns (1980) and Bolsover and Brown (1985) have also presented evidence for a role for calcium in excitation, based on desensitization of the light response after depletion of cellular calcium and facilitation in the presence of high extracellular calcium concentrations. It remains to be seen whether calcium buffers abolish the effects of other isomers of IP_3 that may be produced upon illumination of ventral photoreceptors and which therefore may function as messengers of an alternative pathway of excitation. Inositol 1,3,4 trisphosphate

(Irvine et al., 1985), or cyclic compounds such as inositol 1:2-cyclic 4,5-trisphosphate (Wilson et al., 1985), are possible candidates. The purification of the samples used in our experiments by paper chromatography and their identification against standard samples (Irvine et al., 1984) make contamination of our samples with these other isomers unlikely.

We cannot, however, completely rule out the possibility that excitation by light is solely mediated through the release of calcium via release of IP_3 from the membrane. IP_3 released by light might have access to a confined area that is inaccessible to IP_3 injected into the bulk of the cell. EGTA might fail to abolish the light response because it is ineffective in buffering calcium that is released by light into this confined area close to the channels. EGTA might be excluded from this space or it may become saturated with the released calcium. The slowing of the decline of the light response would then have to be attributed to slow release of calcium from the buffer or slowed reuptake or diffusion of calcium from the confined space owing to the entrapment in the confined site of saturated buffer. Injected IP_3 would then have to be presumed to act at a site more accessible to EGTA in order to explain the ability of EGTA to block the response to injected IP_3. The structural basis for the confined compartment could be the small gap between the base of the microvilli and the network of submicrovillar cisternae of smooth endoplasmic reticulum present in ventral photoreceptors (Calman and Chamberlain, 1982; Walz and Fein, 1983), as well as in other invertebrate photoreceptors (Walz, 1982). This smooth endoplasmic reticulum accumulates calcium in an ATP-dependent manner (Walz, 1982; Walz and Fein, 1983) and it could be the store from which calcium is released by light. IP_3 could be released into this gap by light, and could release calcium from sites facing the microvilli, while injected IP_3 would have access only to calcium release sites on the side of the cisternae facing the cell interior.

Is Light Adaptation Mediated by IP_3?

The results of this article are consistent with the mediation of light adaptation through the light-induced release of IP_3. There is extensive evidence implicating calcium release by light as the cause of light adaptation. Both ionophoretic and pressure injection of calcium desensitize the receptor (Lisman and Brown, 1972; Payne et al., 1986) in a manner that is quantitatively similar to the desensitization caused by light adaptation (Fein and Lisman, 1975; Fein and Charlton, 1977). Bright flashes induce a rapid rise in Ca_i (Brown and Blinks, 1974), and steady illumination causes a maintained increase in Ca_i that is correlated with the desensitization accompanying the steady adapting light (Levy and Fein, 1985).

It is attractive to propose that the release of calcium by light that initiates adaptation is mediated by the release of IP_3 or some closely related compound. Lisman and Strong (1979) have shown that light adaptation is initiated after the absorption of light by rhodopsin. Since light appears to release calcium from an intracellular compartment (Brown and Blinks, 1974), IP_3 could act as a messenger between the plasma membrane where rhodopsin is situated and a nonmitochondrial internal store such as the submicrovillar cisternae. The desensitization caused by IP_3 is spatially localized within the R-lobe (Fein et al., 1984), as is light adaptation (Payne and Fein, 1983). This article shows that EGTA suppresses the

adaptation caused by IP_3, just as it tends to suppress light adaptation (Lisman and Brown, 1975), and that the rise in intracellular calcium caused by IP_3 is rapid enough to mediate light adaptation.

REFERENCES

Bacigalupo, J., and J. E. Lisman. 1983. Single channel currents activated by light in *Limulus* ventral photoreceptors. *Nature.* 304:268–270.

Berridge, M. J. 1983. Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyse polyphosphoinositides instead of phosphatidylinositol. *Biochemical Journal.* 212:849–858.

Berridge, M. J., and R. F. Irvine. 1984. Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature.* 312:315–321.

Bolsover, S. R., and J. E. Brown. 1985. Calcium ion, an intracellular messenger of light adaptation, also participates in excitation of *Limulus* photoreceptors. *Journal of Physiology.* 364:381–393.

Brown, J. E., and J. R. Blinks. 1974. Changes in intracellular free calcium during illumination of invertebrate photoreceptors. Detection with aequorin. *Journal of General Physiology.* 64:643–665.

Brown, J. E., and L. J. Rubin. 1984. A direct demonstration that inositol trisphosphate induces an increase in intracellular calcium in *Limulus* photoreceptors. *Biochemical and Biophysical Research Communications.* 125:1137–1142.

Brown, J. E., L. J. Rubin, A. J. Ghalayini, A. L. Tarver, R. F. Irvine, M. J. Berridge, and R. E. Anderson. 1984. Myo-inositol polyphosphate may be a messenger for visual excitation in *Limulus* photoreceptors. *Nature.* 311:160–162.

Calman, B. G., and S. C. Chamberlain. 1982. Distinct lobes of *Limulus* ventral photoreceptors. II. Structure and ultrastructure. *Journal of General Physiology.* 80:839–862.

Corson, D. W., A. Fein, and R. Payne. 1984. Detection of an inositol 1,4,5 trisphosphate-induced rise in intracellular free calcium with aequorin in *Limulus* ventral photoreceptors. *Biological Bulletin.* 167:524. (Abstr.)

Fein, A., and J. S. Charlton. 1977. A quantitative comparison of the effects of intracellular calcium injection on the photoresponse of *Limulus* ventral photoreceptors. *Journal of General Physiology.* 70:591–600.

Fein, A., and J. E. Lisman. 1975. Localized desensitization of *Limulus* photoreceptors produced by light or intracellular calcium ion injection. *Science.* 187:1094–1096.

Fein, A., R. Payne, D. W. Corson, M. J. Berridge, and R. F. Irvine. 1984. Photoreceptor excitation and adaptation by inositol 1,4,5 trisphosphate. *Nature.* 311:157–160.

Grado, C., and C. E. Ballou. 1961. Myo-inositol phosphates obtained by alkaline hydrolysis of beef brain polyphosphoinositide. *Journal of Biological Chemistry.* 236:54–60.

Irvine, R. F., R. E. Anderson, L. J. Rubin, and J. E. Brown. 1985. Inositol 1,3,4 trisphosphate

concentration is changed by illumination of *Limulus* ventral photoreceptors. *Biophysical Journal.* 47:38a. (Abstr.)

Irvine, R. F., K. D. Brown, and M. J. Berridge. 1984. Specificity of inositol trisphosphate-induced calcium release from permeabilized Swiss-mouse 3T3 cells. *Biochemical Journal.* 222:269–272.

Levy, S., and A. Fein. 1985. Relationship between light sensitivity and intracellular free calcium in *Limulus* ventral photoreceptors. *Journal of General Physiology.* 85:805–841.

Lisman, J. E., and J. E. Brown. 1972. The effects of intracellular iontophoretic injection of calcium and sodium ions on the light response of *Limulus* ventral photoreceptors. *Journal of General Physiology.* 59:701–719.

Lisman, J. E., and J. E. Brown. 1975. Effects of intracellular injection of calcium buffers on light adaptation in *Limulus* ventral photoreceptors. *Journal of General Physiology.* 66:489–506.

Lisman, J. E., and J. A. Strong. 1979. The initiation of excitation and light adaptation in *Limulus* ventral photoreceptors. *Journal of General Physiology.* 73:219–243.

Michell, R. H. 1975. Inositol phospholipids and cell surface receptor function. *Biochemica et Biophysica Acta.* 415:81–147.

Millecchia, R., and A. Mauro. 1969. The ventral photoreceptor cells of *Limulus*. III. A voltage-clamp study. *Journal of General Physiology.* 54:331–351.

Payne, R., D. W. Corson, and A. Fein. 1986. Pressure injection of calcium both excites and adapts *Limulus* ventral photoreceptors. *Journal of General Physiology.* 88:107–126.

Payne, R., and A. Fein. 1984. Modification to the light-response of *Limulus* ventral photoreceptors by intracellular Ca/EGTA. *Biophysical Journal.* 45:293a. (Abstr.)

Payne, R., and A. Fein. 1983. Localized adaptation within the rhabdomeral lobe of *Limulus* ventral photoreceptors. *Journal of General Physiology.* 81:767–769.

Payne, R., and A. Fein. 1986. The initial response of *Limulus* ventral photoreceptors to bright flashes. Released calcium as a synergist to excitation. *Journal of General Physiology.* 87:243–269.

Payne, R., A. Fein, and D. W. Corson. 1984. A rise in intracellular calcium is necessary and perhaps sufficient for photoreceptor excitation and adaptation by inositol 1,4,5 trisphosphate. *Biological Bulletin.* 167:531. (Abstr.)

Rubin, L. J., and J. E. Brown. 1985. Intracellular injection of calcium buffers blocks IP$_3$-induced but not light-induced electrical responses of *Limulus* ventral photoreceptors. *Biophysical Journal.* 47:38a. (Abstr.)

Shimomura O., F. H. Johnson, and Y. Saiga. 1962. Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan *Aequorea*. *Journal of Cellular and Comparative Physiology.* 59:223–240.

Stieve, H., and M. Bruns. 1980. Dependence of bump rate and bump size in *Limulus* ventral nerve photoreceptor on light adaptation and calcium concentration. *Biophysics of Structure and Mechanism.* 6:271–285.

Streb, H., E. Bayerdorffer, W. Haase, R. F. Irvine, and I. Schulz. 1984. Effect of inositol 1,4,5 trisphosphate on isolated subcellular fractions of rat pancreas. *Journal of Membrane Biology.* 81:241–253.

Streb, H., R. F. Irvine, M. J. Berridge, and I. Schultz. 1983. Release of Ca^{2+} from a nonmitochondrial store in pancreatic acinar cells by inositol 1,4,5 trisphosphate. *Nature.* 306:67–69.

Tomlinson, R. V., and C. E. Ballou. 1961. Complete characterization of the myo-inositol

polyphosphates from beef brain phosphoinositide. *Journal of Biological Chemistry.* 236:1902–1906.

Tsien, R. Y. 1980. New calcium indicators and buffers with high selectivity against magnesium and protons. Design, synthesis and properties of prototype structures. *Biochemistry.* 19:2396–2404.

Walz, B. 1982. Ca^{2+}-sequestering smooth endoplasmic reticulum in an invertebrate photoreceptor. I. Intracellular topography as revealed by OsFeCN staining and *in situ* Ca accumulation. *Journal of Cell Biology.* 93:839–848.

Walz, B., and A. Fein. 1983. Evidence for calcium-sequestering smooth ER in *Limulus* ventral photoreceptors. *Investigative Ophthalmology and Visual Science.* 24(Suppl.):281.

Wilson, D. B., T. M. Connolly, T. E. Bross, P. W. Majerus, W. R. Sheman, A. W. Tyler, L. J. Rubin, and J. E. Brown. 1985. Isolation and characterization of the inositol cyclic phosphate products of polyphosphoinositide cleavage by phospholipase-C. *Journal of Biological Chemistry.* 260:13496–13501.

Wong, F. 1978. Nature of light-induced conductance changes in ventral photoreceptors of *Limulus. Nature.* 276:76–79.