Injection of Inositol Trisphosphorothioate into *Limulus* Ventral Photoreceptors Causes Oscillations of Free Cytosolic Calcium

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ABSTRACT *Limulus* ventral photoreceptors contain calcium stores sensitive to release by D-*myo*-inositol 1,4,5 trisphosphate ($InsP_3$) and a calcium-activated conductance that depolarizes the cell. Mechanisms that terminate the response to $InsP_3$ were investigated using nonmetabolizable DL-*myo*-inositol 1,4,5 trisphosphorothioate ($InsPS_3$). An injection of 1 mM $InsPS_3$ into a photoreceptor's light-sensitive lobe caused an initial elevation of cytosolic free calcium ion concentration (Ca_i) and a depolarization lasting only 1–2 s. A period of densensitization followed, during which injections of $InsPS_3$ were ineffective. As sensitivity recovered, oscillations of membrane potential began, continuing for many minutes with a frequency of 0.07–0.3 Hz. The activity of $InsPS_3$ probably results from the D-stereoisomer, since L-$InsP_3$ was much less effective than $InsP_3$. Injections of 1 mM $InsP_3$ caused an initial depolarization and a period of densensitization similar to that caused by 1 mM $InsPS_3$, but no sustained oscillations of membrane potential. The initial response to $InsPS_3$ or $InsP_3$ may therefore be terminated by desensitization, rather than by metabolism. Metabolism of $InsP_3$ may prevent oscillations of membrane potential after sensitivity has recovered. The $InsPS_3$-induced oscillations of membrane potential accompanied oscillations of Ca_i and were abolished by injection of ethyleneglycol-bis (β-aminoethyl ether)-*N*, *N'*-tetraacetic acid. Removal of extracellular calcium reduced the frequency of oscillation but not its amplitude. Under voltage clamp, oscillations of inward current were observed. These results indicate that periodic bursts of calcium release underly the oscillations of membrane potential. After each burst, the sensitivity of the cell to injected $InsP_3$ was greatly reduced, recovering during the interburst interval. The oscillations may, therefore, result in part from a periodic variation in sensitivity to a constant concentration of $InsPS_3$. Prior injection of calcium inhibited depolarization by $InsPS_3$, suggesting that feedback inhibition of $InsPS_3$-induced calcium release by elevated Ca_i may mediate desensitization between bursts and after injections of $InsPS_3$.

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INTRODUCTION

D-*myo*-inositol 1,4,5 trisphosphate ($InsP_3$) is thought to mediate the ability of light to release calcium from endoplasmic reticulum (ER) within invertebrate microvillar photoreceptors (Brown et al., 1984; Fein et al., 1984; Baumann and Walz, 1989). A brief (30–200 ms) injection of $InsP_3$ into the light-sensitive lobe of a *Limulus* ventral photoreceptor usually causes a transient rise in cytosolic free calcium ion concentration (Ca_i), lasting 1–2 s (Brown and Rubin, 1984; Payne et al., 1986*b*). The rise in Ca_i is accompanied by a depolarization which results from a calcium-activated increase in the sodium conductance of the photoreceptor's plasma membrane (Payne et al., 1986*a*). This sodium conductance has similar properties to that activated by light (Brown et al., 1984; Fein et al., 1984; Payne et al., 1986*a*), suggesting that the release of calcium by $InsP_3$ participates in phototransduction.

Several processes might contribute to the rapid termination of the release of calcium by $InsP_3$. Two of these might reduce the concentration of $InsP_3$ at the injection site. First, $InsP_3$ might be metabolized to inactive products, such as inositol bisphosphate ($InsP_2$; Berridge and Irvine, 1984). Secondly, dilution of $InsP_3$ into the cell volume may reduce its concentration below the threshold for calcium release. Two other processes may act rapidly to reduce the effectiveness of $InsP_3$ in releasing calcium. First, the $InsP_3$-sensitive ER might become rapidly depleted of calcium (Berridge and Irvine, 1989). Secondly, the calcium release mechanism might become less sensitive to $InsP_3$ through feedback inhibition by elevated Ca_i (Worley et al., 1987; Baumann and Walz, 1989; Ogden et al., 1990; Parker and Ivorra, 1990; Payne et al., 1990) or by some other mechanism such as phosphorylation of the $InsP_3$ receptor (Supattapone et al., 1988).

Invertebrate microvillar photoreceptors contain enzymes that rapidly dephosphorylate $InsP_3$ to inactive $Ins(1,4)P_2$ (Trowell, 1988; Wood et al., 1990). Wood et al. (1990) estimate that an initial concentration of $InsP_3$ of 10μM within the outer segment of a squid photoreceptor would be metabolized to $InsP_2$ with a half-time of 5 s. The role of the metabolism of $InsP_3$ in terminating $InsP_3$-induced calcium release can be investigated by injecting an analogue of $InsP_3$ that is resistant to metabolism, DL-*myo*-inositol 1,4,5 trisphosphorothioate ($InsPS_3$; Cooke et al., 1987*a*; Nahorski and Potter, 1989; Taylor et al., 1989). If rapid metabolism of $InsP_3$ terminates calcium release then the injection of $InsPS_3$ should prolong calcium release. We find that calcium release after injection of $InsPS_3$ is prolonged, but it is not continuous. A large, transient elevation of Ca_i, similar to that caused by $InsP_3$, is followed by a period of insensitivity to further injections of $InsPS_3$. As sensitivity returns, a series of oscillatory bursts of calcium release begin and continue for at least tens of minutes. Rapid desensitization of the mechanism for calcium release may account for both the decline of the initial transient rise in Ca_i and the transience of each oscillatory elevation of Ca_i.

In addition to providing insight into the mechanism that releases calcium, the use of $InsPS_3$ provides an important test of the hypothesis that $InsP_3$ participates in the electrical response of the photoreceptor to light (Brown et al., 1984; Fein et al., 1984). If $InsP_3$ is the only messenger of phototransduction in *Limulus* photoreceptors, then injection of $InsPS_3$ should mimic steady illumination. The oscillatory bursts of

depolarization that we observe after injection of $InsPS_3$ clearly do not mimic the sustained depolarization produced by steady illumination. Our results do not, therefore, support the proposal that $InsP_3$ is the only messenger that mediates the electrical response to light.

MATERIALS AND METHODS

Experimental Procedures

Conventional methods of intracellular recording and for stimulating ventral nerve photoreceptors were used, as described in detail elsewhere (Millecchia and Mauro, 1969; Fein and Charlton, 1977; Payne et al., 1990). Cells were stimulated with white light from a 100–W quartz-halogen source which was passed through a heat filter, neutral density (ND) filters, and a shutter before being focused onto the specimen plane. The intensity of light at the specimen, with no intervening ND filters, was 80 mW/cm^2. Light intensities are quoted in this paper as log units of attenuation relative to this intensity. In order to view the preparation with an infrared-sensitive TV camera, cells were also continuously illuminated by an infrared beam, created by passing a second beam of light from the quartz-halogen lamp through an infrared filter before focusing it onto the specimen.

Rapid pressure injection of substances into cells through single and double-barreled ("theta glass") micropipette was achieved as previously described (Corson and Fein, 1983; Payne et al., 1990).

Observation of Aequorin Luminescence

Light from the preparation was collected by an objective lens (L25xFL, 0.36 NA, E. Leitz, Wetzlar, Federal Republic of Germany) and projected onto a dichroic mirror (DC675LP, Omega Optical Inc., Brattleboro, VT) mounted at 45° to the light beam. Infrared light passed through the dichroic mirror and was focused onto the infrared-sensitive TV camera. Visible light, including aequorin luminescence, which was reflected from the dichroic mirror, was focused onto the photocathode of a photomultiplier tube (R464, Hamamatsu Corp., Bridgewater, NJ). Transistor–transistor logic (TTL) pulses from the output of a photon-counting amplifier/discriminator (3470/AD6, Pacific Instruments, Inc., Concord, CA) were counted, placed into time bins, and continuously displayed by an IBM AT computer. Aequorin luminescence is expressed in the text as counts per second (cps)

Chemicals and Solutions

DL-$InsPS_3$ was synthesized as described previously (Cooke et al., 1987*a*) and purified by ion exchange chromatography on diethylaminoethyl (DEAE) Sephadex A-25. Reference to $InsPS_3$ implies the racemate unless indicated. L-$InsP_3$ was synthesized from resolved D-1,2,4,-tri-*O*-benzyl-*myo*-inositol (Gigg et al., 1987) essentially as described for DL-$InsP_3$ (Cooke et al., 1987*b*). All synthetic inositol polyphosphates were used as the triethylammonium salts. D-$InsP_3$ was obtained from Calbiochem-Behring Corp., San Diego, CA, as the trilithium salt.

All chemicals injected into the cells were dissolved in carrier solution (100 mM potassium aspartate, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2 ethane sulfonic acid [HEPES] pH 7.0). Potassium aspartate and aspartic acid were obtained from Sigma Chemical Co., St. Louis, MO. All inorganic reagents were of analytical grade. Calcium aspartate was made by the addition of stoichiometric amounts of $Ca(OH)_2$ to aspartic acid. Cells were normally bathed in artificial seawater (ASW) that contained 435 mM NaCl, 10 mM $CaCl_2$, 10 mM KCl, 20 mM $MgCl_2$, 25

mM $MgSO_4$, and 10 mM HEPES, pH 7.0 0Ca-EGTA ASW was made by replacing the $CaCl_2$ with 1 mM EGTA obtained from Sigma Chemical Co.

Recombinant aequorin was the generous gift of Dr. O. Shimomura (Marine Biological Laboratory, Woods Hole, MA), Dr. S. Inouye (Chisso Chemical Corp., Yokohama, Japan), and Dr. Y. Kishi (Department of Chemistry, Harvard University, Cambridge MA). Recombinant aequorin was made by incubating recombinant apo-aequorin (Inouye et al., 1985, 1989) with coelenterazine (Kishi et al., 1972; Musicki et al., 1986). For microinjection, the aequorin was dissolved at a concentration of 6.7 mg/ml in carrier solution containing 100 μM EGTA.

RESULTS

Injection of $InsPS_3$ Causes an Immediate Transient Depolarization Followed by Oscillatory Bursts

Ventral photoreceptors are clearly divided into two or more lobes (Calman and Chamberlain, 1982; Stern et al., 1982), one or more light-sensitive rhabdomeral (R) lobes and an arhabdomeral (A) lobe that is insensitive to light. The sizes of the R and A lobes are highly variable from cell to cell, with diameters ranging from 40 to 100 μm and centers placed between 40 and 100 μm apart. The R lobe contains the $InsP_3$-sensitive ER (Payne and Fein, 1987) and the sites at which calcium acts to depolarize the photoreceptor (Payne et al., 1986*a*).

A single, brief injection of 1 mM $InsPS_3$ delivered, in darkness, from a micropipette that impaled the R lobe of a photoreceptor caused a large immediate depolarization lasting <2 s, followed by a period of reduced activity and then a series of bursts of depolarization (Fig. 1 *A*). The bursts continued for several minutes (Fig. 1 *B*). A further injection of $InsPS_3$ delivered during the bursts of depolarization (Fig. 1 *B*, first vertical arrow) caused another large immediate depolarization followed by a period of inactivity.

After a third injection of $InsPS_3$ (Fig. 1 *B*, second vertical arrow), the bursts developed a stable frequency of occurrence and continued for 41 min (Fig. 1 *C*), after which we terminated the recording. We shall refer to these regular bursts as "oscillations" of membrane potential. The frequency of the oscillations varied from cell to cell from 0.3 to 0.07 Hz. The regularity of occurrence of the bursts of depolarization also varied from cell to cell. Not every cell injected with $InsPS_3$ developed stable oscillations of membrane potential. Many other cells exhibited irregularly occurring bursts of depolarization, the bursts having a similar mean frequency of occurrence to the oscillations. Like the oscillations, these irregularly-occurring bursts continued for many minutes after the injection. Out of 65 cells injected with $InsPS_3$, oscillations of membrane potential were recorded from 25 cells, while irregularly-occurring bursts of depolarization were recorded from the remainder.

Some photoreceptors were impaled with a second micropipette through which current was passed to clamp the membrane potential to its normal resting level. Once impaled, cells were injected with sufficient $InsPS_3$ to elicit oscillations of membrane potential. Voltage clamp of these photoreceptors at their resting potential (between -40 and -60 mV) did not alter the frequency of the oscillations, now of inward current rather than depolarization (Fig. 2). Modulation of channel activity by

membrane potential is therefore not necessary for the oscillations to occur. Similar results to those of Fig. 2 were obtained from five other cells.

The Site of Action of $InsP_3$ Is Stereospecific

Because the stock of $InsPS_3$ was a racemate (Cooke et al., 1987*a*; Taylor et al., 1989), we investigated the stereospecificity of the action of $InsP_3$ in order to determine which

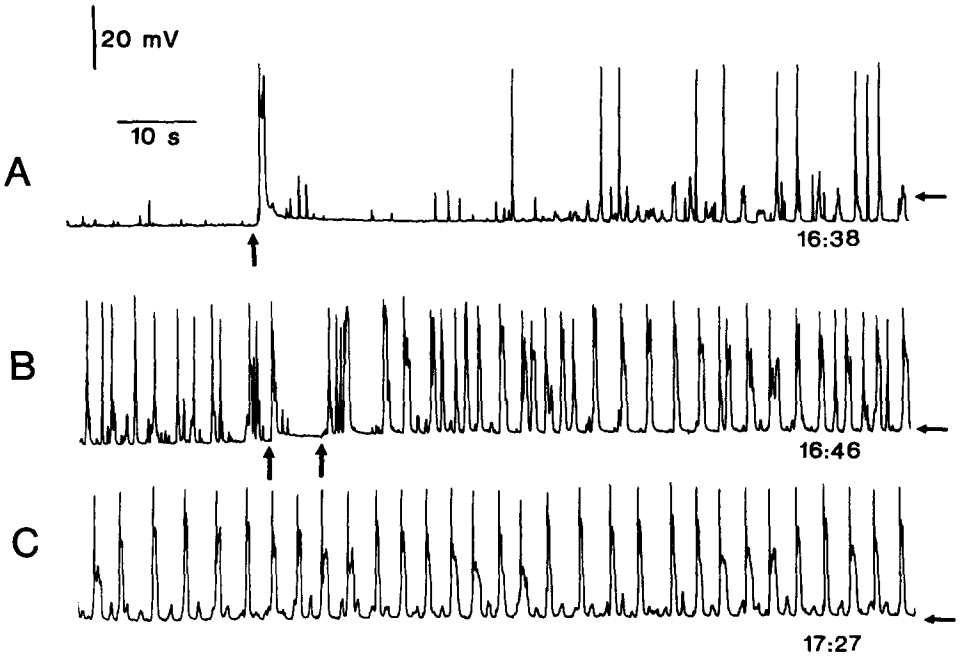

FIGURE 1. The injection of $InsPS_3$ into a ventral photoreceptor causes an immediate depolarization, followed by oscillations of membrane potential. The photoreceptor was impaled in its R lobe with a micropipette containing 1 mM $InsPS_3$. The time (in hours:minutes) at which each recording began is shown below each trace, on the left. The duration of the injections was 300 ms, pressure 30 psi. The horizontal arrow to the right of each trace indicates an absolute membrane potential of −45 mV. All traces were recorded from the same cell. (*A*) Membrane potential recorded during an injection, at the vertical arrow, of 1 mM $InsPS_3$. The injection caused an immediate depolarization, lasting 1–2 s followed by delayed bursts of depolarization. (*B*) 8 min after the recording shown in *A*, the bursts of depolarization persisted. Two subsequent injections of $InsPS_3$ (vertical arrows) resulted in immediate depolarizations, followed by regular oscillations of membrane potential. (*C*) The oscillations of membrane potential persisted for 41 min after the recording shown in *B*.

of the isomers was likely to be effective in releasing calcium. We injected 1 mM L-$InsP_3$ (Strupish et al., 1988) into the R lobe of a ventral photoreceptor through a double-barreled electrode which held either 1 mM D-$InsP_3$ or 1 mM $InsPS_3$ in the other barrel. Injections of L-$InsP_3$ were much less effective than those of either D-$InsP_3$ or $InsPS_3$ in depolarizing the photoreceptor and did not elicit oscillatory

bursts of depolarization. The mean immediate depolarization resulting from an injection of L-$InsP_3$ into seven cells was 2.3 ± 2 mV (SEM) whereas that to D-$InsP_3$ was 40 ± 5 mV.

The Relative Effectiveness of $InsPS_3$ and $InsP_3$ Depends on the Site of Their Injection

We compared the responses of the photoreceptor to injections of 1 mM $InsP_3$ with those to 1 mM $InsPS_3$. We also determined the dependence of the effectiveness of the

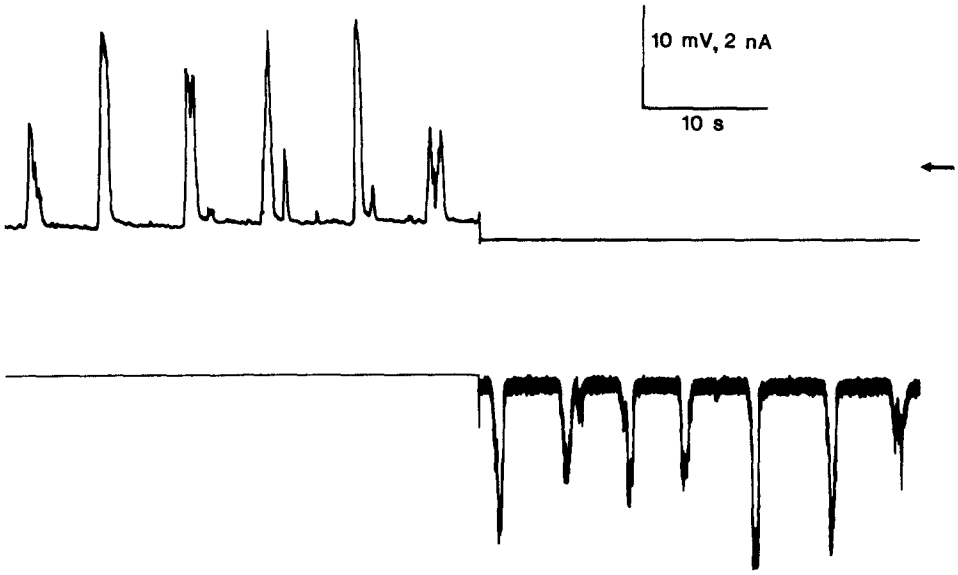

FIGURE 2. Voltage clamp of a photoreceptor during $InsPS_3$-induced oscillations of membrane potential resulted in oscillations of inward current. A photoreceptor was first injected with sufficient $InsPS_3$ to induce sustained oscillations of membrane potential. Oscillations of membrane potential were first recorded before voltage clamp as shown by the upper (voltage) trace on the right-hand side of the figure. The voltage-clamp circuitry was then activated to clamp the membrane potential at −52 mV and oscillations of current were recorded as shown by the lower (current) trace. Current flowing into the cell is shown as a negative deflection of the trace. The horizontal arrow to the right of the voltage trace indicates an absolute membrane potential of −45 mV.

injections on their location within the photoreceptor. $InsP_3$ has been shown to be less effective when injected into the A lobe rather than into the R lobe (Fein et al., 1984). We wished to determine whether this was due to metabolism of $InsP_3$ while *en route* from the A lobe to the ER in the R lobe.

Injections of $InsPS_3$ and $InsP_3$ into the R lobe. We filled a double-barreled micropipette with 1 mM $InsP_3$ in one barrel and 1 mM $InsPS_3$ in the other. A photoreceptor was impaled with this micropipette in either its A or R lobe, placement being judged by the different visual appearance of the lobes (Stern et al., 1982). At

the end of the experiment, the identification of the lobe was confirmed by scanning the photoreceptor with a 20-μm-diam spot of light, and recording the depolarization produced by brief flashes of light. Sensitivity to light is greatest in the R lobe and least in the A lobe (Stern et al., 1982).

A series of five brief injections were delivered to either barrel of the micropipette. When placed in the R lobe (Fig. 3 *A*), the response to the first injection of $InsP_3$ consisted of an immediate, transient depolarization. Subsequent injections of $InsP_3$,

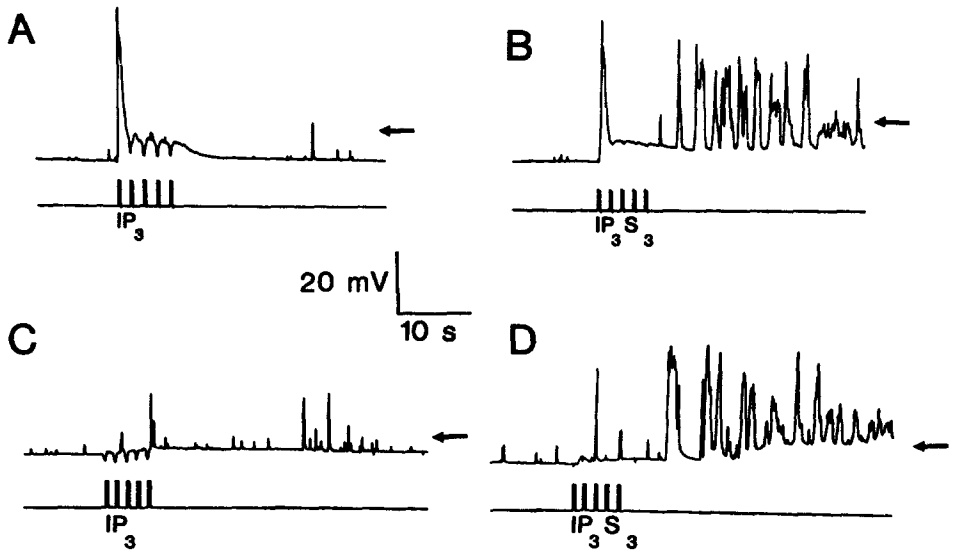

FIGURE 3. Relative effectiveness of injections of $InsP_3$ or $InsPS_3$ when injected into the R lobe (*A* and *B*) or A lobe (*C* and *D*) of two ventral photoreceptors. (*A* and *B*) Membrane potential during injections of (*A*) 1 mM $InsP_3$ and (*B*) 1 mM $InsPS_3$ delivered into the R lobe of a photoreceptor through a double-barreled micropipette. $InsP_3$ and $InsPS_3$ produce a similar immediate depolarization, but no oscillations follow the injection of $InsP_3$. (*C* and *D*) Membrane potential during injections of (*C*) 1 mM $InsP_3$ and (*D*) 1 mM $InsPS_3$ delivered into the A lobe of another photoreceptor. $InsPS_3$ is much more effective than $InsP_3$ in producing delayed oscillations of membrane potential. The lower trace in each record shows the time and duration of the pressure pulse delivered to the micropipette. The transient negative deflection after each injection of $InsP_3$ after the first is an injection artifact resulting from the use of the barrel containing $InsP_3$ to record membrane potential. All injections were of duration 300 ms, pressure 50 psi. The horizontal arrow to the right of each trace indicates an absolute membrane potential of −45 mV.

delivered between 2 and 8 s after the first were ineffective in causing another transient depolarization. Sensitivity to injections of $InsP_3$ recovered completely within 1 min (not shown). The response to $InsPS_3$ was more complex (Fig. 3 *B*). An immediate, transient depolarization, very similar to that caused by $InsP_3$, immediately followed the injection. As for injections of $InsP_3$, a period of desensitization followed, during which subsequent injections were ineffective. However, 11 s later, bursts of

depolarization developed and continued for the next 15 min when the recording was terminated.

Out of a total of eight cells investigated, in all cases the immediate transient depolarizations after the first injections of either $InsP_3$ or $InsPS_3$ were similar in amplitude and time course. The severity of the desensitization after the first injections varied from cell to cell. For four of the eight cells, injections of $InsPS_3$ or $InsP_3$ after the first were entirely ineffective (as in Fig. 3 *B*). In the other cells,

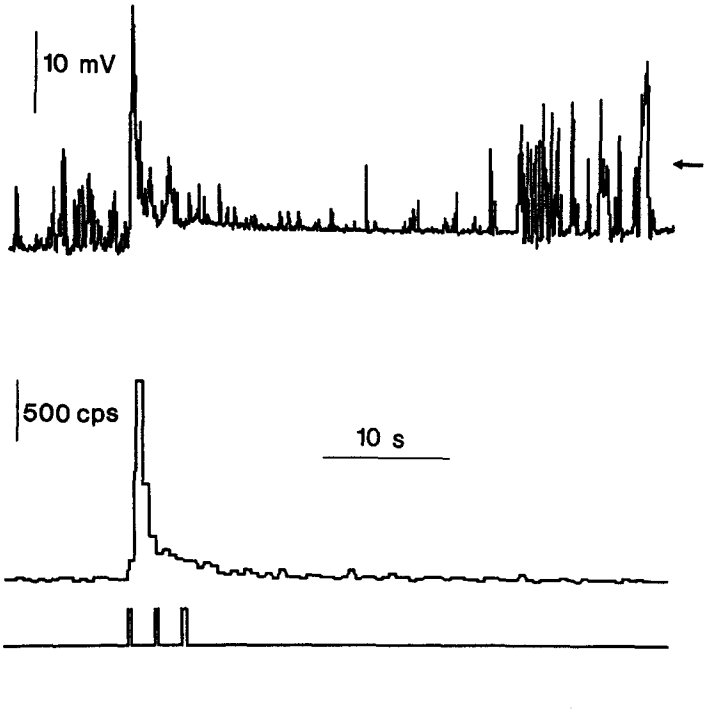

FIGURE 4. Only the first of a series of injections of 1 mM $InsPS_3$ into the R lobe of a ventral photoreceptor results in a significant elevation of Ca_i, as detected by aequorin. Depolarization (top trace) and aequorin luminescence (middle trace) were recorded during a series of three injections of 1 mM $InsPS_3$ (monitored by the bottom trace). Note the lingering "tail" of aequorin luminescence that follows the injections. The aequorin luminescence was counted into bins of 500 ms in duration. The injections were of duration 200 ms, 50 psi. The horizontal arrow to the right of the voltage trace indicates an absolute membrane potential of −45 mV.

injections after the first resulted in transient depolarizations which were much smaller than that caused by the first injection and which were delayed with respect to the time of injection (for example, Fig. 4). After the period of desensitization, bursts of depolarization were irreversibly elicited by injections of 1 mM $InsPS_3$ into the R lobe of seven of the cells. Injection of $InsPS_3$ into the remaining cell resulted in an irreversible desensitization of the cell to both light and $InsPS_3$. In only one of the eight cells were bursts of depolarization elicited by injections of $InsP_3$ and in this case,

the bursts died out within 45 s. Addition of 10 mM triethylammonium aspartate (the counterion to $InsPS_3$; see Materials and Methods) to the solution of 1 mM $InsP_3$ resulted in similar responses to injections into eight cells as were observed after injections of 1 mM $InsP_3$ alone.

Injections of $InsPS_3$ and $InsP_3$ into the A lobe. When the micropipette was placed in the A lobe of another photoreceptor, injections of $InsP_3$ elicited only a few small bursts of depolarization (Fig. 3 *C*). Injections of $InsPS_3$, however, elicited a striking series of bursts of depolarization after a delay of 11 s between the injection of $InsPS_3$ and the first burst (Fig. 3 *D*). For five other cells, the delay varied from 4 to 13 s. In all cells, the bursts of depolarization continued for many minutes after the injections. We conclude that $InsPS_3$ is much more effective than $InsP_3$ when injected into the A lobe and that the metabolism of $InsP_3$ may therefore play a role in limiting its spread within the photoreceptor.

Desensitization of the Response to $InsPS_3$ Results from a Decreased Ability of $InsPS_3$ to Elevate Ca_i

The desensitization of the response to injections of $InsP_3$ that follow a first, effective injection has been shown to be caused by an inability of $InsP_3$ to release calcium (Payne et al., 1990). We wished to determine whether the same holds for the response to $InsPS_3$. We therefore impaled a photoreceptor with a micropipette containing aequorin (Shimomura et al., 1962; Brown and Blinks, 1974) and injected 10–100 pl of aequorin solution (see Materials and Methods) before impalement with another micropipette containing 1 mM $InsPS_3$. Changes in Ca_i were then monitored during a series of three injections of 1 mM $InsPS_3$, delivered to the R lobe (Fig. 4). The first injection resulted in an immediate depolarization of 32 mV which reached its peak 400 ms after the injection began. This immediate depolarization was accompanied by a large increase in aequorin luminescence, the peak emission being 1,618 cps. The second injection, delivered 2 s after the first, failed to elicit an immediate depolarization. However, a small delayed depolarization reaching a peak 1.4 s after the beginning of the second injection is evident. No significant increase in aequorin luminescence followed the second injection. The third injection of $InsPS_3$, delivered 4 s after the first, failed to significantly depolarize the photoreceptor or to detectably increase the aequorin luminescence.

After the first injection of $InsPS_3$, the aequorin luminescence remained elevated, creating a "tail" of luminescence that declined throughout the period of desensitization. The mean aequorin luminescence before the first injection was 24 cps. Before the second injection, it was 374 cps and before the third it had declined somewhat to 190 cps. The mean aequorin luminescence had declined to 40 cps by the time at which the bursts of depolarization began.

The period of desensitization and inactivity is therefore one in which Ca_i remains elevated. Similar results were obtained from six other cells. A transient depolarization, aequorin luminescence and subsequent desensitization were also recorded when cells bathed in 0Ca-EGTA ASW were injected with $InsPS_3$, indicating that, as for $InsP_3$ (Brown and Rubin, 1984; Payne et al., 1986*b*) the elevation of Ca_i results from the release of calcium from intracellular stores.

A Prior Injection of Calcium Desensitizes the Response to DL-InsPS$_3$

The experiment of Fig. 4 demonstrates that the period of desensitization to $InsPS_3$ is accompanied by a sustained elevation of Ca_i. Previous work has shown that prior elevation of Ca_i inhibits calcium release by subsequent injections of $InsP_3$ (Payne et al., 1990). We thought it likely that the same mechanism might account for the

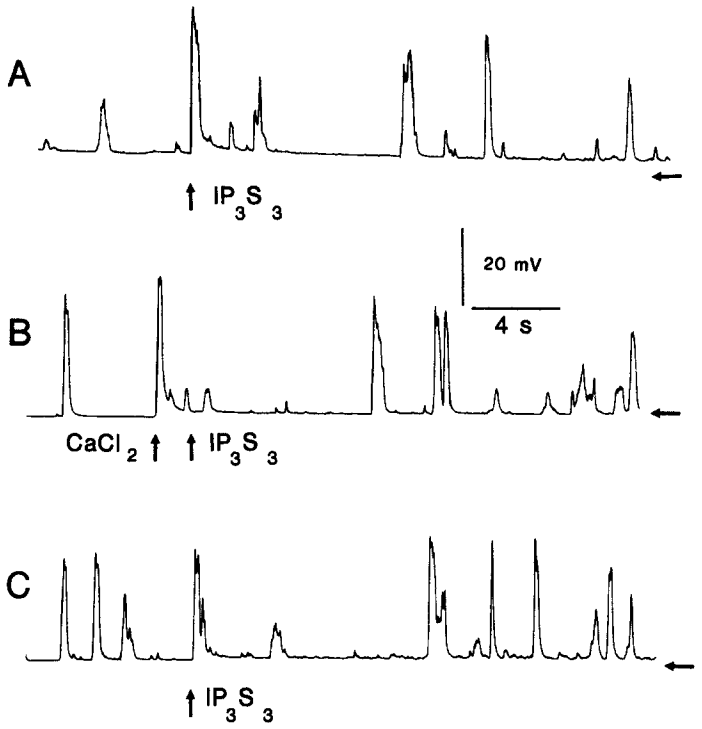

FIGURE 5. Prior injection of calcium inhibits the immediate depolarization resulting from injection of 1 mM $InsPS_3$ into a ventral photoreceptor. (*A*) Membrane potential recorded during an injection of 1 mM $InsPS_3$ into the R lobe of a ventral photoreceptor. The injection, duration 100 ms, pressure 20 psi, was delivered at the vertical arrow. The injection of $InsPS_3$ causes a large immediate depolarization. (*B*) Membrane potential recorded during an injection of 1 mM $CaCl_2$ (first vertical arrow; duration 100 ms, pressure 20 psi), followed by an injection of 1 mM $InsPS_3$ (second vertical arrow). The injection of $CaCl_2$ causes a large immediate depolarization, but the injection of $InsPS_3$ is ineffective. (*C*) 2 min after the recording in *B*, another injection of $InsPS_3$ was delivered and again produced a large immediate depolarization. The horizontal arrow to the right of each trace indicates an absolute membrane potential of −45 mV. All traces were recorded from the same cell.

period of desensitization to $InsPS_3$. To test this hypothesis directly, we impaled ventral photoreceptors with a double-barreled micropipette containing 1 mM $CaCl_2$ in one barrel and 1 mM $InsPS_3$ in the other. We injected a brief pulse of $CaCl_2$ 1.5 s before one of $InsPS_3$. Fig. 5 *B* shows that the injection of calcium caused a transient depolarization, indicating a large elevation of Ca_i, followed by a suppression of the

immediate response to the subsequent injection of $InsPS_3$ (compare Fig. 5 *B* with Fig. 5, *A* and *C*). Similar results were obtained in six cells injected with 1 mM calcium aspartate and in one other cell injected with 1 mM $CaCl_2$. Injection of five cells with carrier solution 1.5 s before an injection of $InsPS_3$ had no significant effect on the $InsPS_3$-induced depolarization.

The $InsPS_3$-induced Oscillations in Membrane Potential Are Accompanied by Oscillations of Ca_i

Photoreceptors were impaled with a micropipette containing aequorin and injected with 10–100 pl of aequorin solution (See Materials and Methods) before impalement with another micropipette containing 1 mM $InsPS_3$. Sufficient 1 mM $InsPS_3$ was then injected to initiate spontaneous oscillatory bursts of depolarization (Fig. 6). The larger and longer bursts of depolarization were each accompanied by a small burst of aequorin luminescence indicating a small, or highly localized, rise in Ca_i (Fig. 6 *A*). Shorter bursts, of amplitude <20 mV were not accompanied by detectable aequorin signals, possibly owing to the long (500 ms) integration time used in counting photons emitted by the aequorin. The aequorin signals associated with each burst were much smaller than those accompanying the immediate depolarization that followed the first of a series of injections of 1 mM $InsPS_3$ delivered to the same cell during the period of oscillatory activity (Fig. 6 *B*). Note that, as for Fig. 4, the period of suppression of oscillatory activity after the injection of $InsPS_3$ is accompanied by a "tail" of elevated Ca_i. The elevations of Ca_i during the oscillations were also very much smaller than those observed after a saturating light flash (Fig. 6 *C*). We conclude that the small elevations of Ca_i that accompany the oscillations of depolarization are probably caused by the release of only a fraction of the calcium stored within the photoreceptor. Similarly small oscillations of aequorin luminescence were observed in four other cells injected with $InsPS_3$.

Injection of the Calcium Buffer EGTA Abolishes the Oscillatory Bursts of Depolarization

Photoreceptors were injected with sufficient $InsPS_3$ to initiate oscillatory bursts of depolarization (Fig. 7 *A*) and then with a single injection of 100 mM EGTA through a second micropipette (Fig. 7 *B*). The injection of EGTA completely suppressed the oscillations. A subsequent injection of 1 mM $InsPS_3$ still elicited a transient depolarization with a complex waveform (Fig. 7 *C*), but did not result in any subsequent oscillatory bursts of depolarization. A further series of six injections of EGTA abolished the response to $InsPS_3$ (Fig. 7 *D*).

We also investigated the effect of the injections of EGTA on the response to prolonged light flashes (Fig. 7, *E–G*). The response to a prolonged light flash before injection of $InsPS_3$ or EGTA is shown in Fig. 7 *E*. The first injection of EGTA, which abolished the oscillations of membrane potential, somewhat reduced the initial transient peak of the light response and increased the sustained plateau (Fig. 7 *F*). The series of EGTA injections that abolished the immediate response to an injection of $InsPS_3$ also abolished the initial transient depolarization caused by the light flash, while the smaller plateau depolarization remained (Fig. 7 *G*). Similar results were obtained from six other cells.

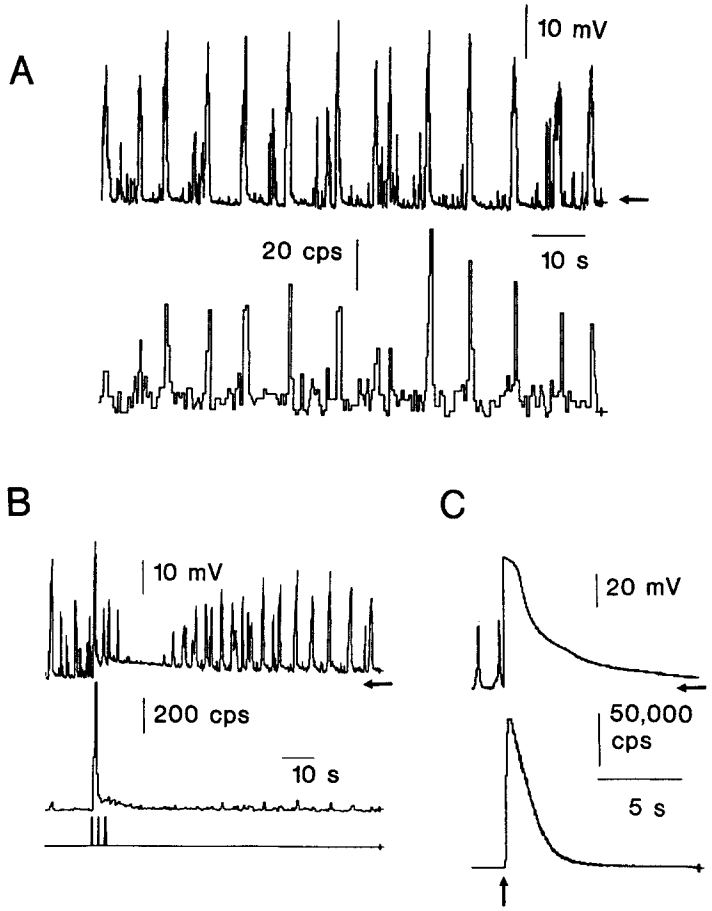

FIGURE 6. The oscillations of membrane potential elicited by injection of 1 mM $InsPS_3$ are accompanied by oscillations of Ca_i and are transiently suppressed by further injections of $InsPS_3$ into the same cell. A bright light flash delivered to the same cell releases much more calcium than does each oscillation. (*A*) Oscillations of membrane potential (upper trace) and aequorin luminescence (lower trace) elicited by prior injection of $InsPS_3$. The aequorin luminescence was counted into 500-ms bins. (*B*) Membrane potential (upper trace) and aequorin luminescence (lower trace) recorded during a series of injections of $InsPS_3$ delivered during the period of oscillatory activity. The first of the injections elicited a large depolarization and a large increase in aequorin luminescence, after which the response to the subsequent injections and the spontaneous oscillations of membrane potential were suppressed. Note the change in scale of the aequorin luminescence trace, as compared to *A*. The aequorin luminescence was counted into 500-ms bins. The injections were of duration 300 ms, pressure 40 psi. (*C*) Membrane potential (upper trace) and aequorin luminescence (lower trace) recorded following a light flash, log intensity = 0, duration 50 ms that was delivered subsequent to the record in *B*. The shutter to the photomultiplier tube was open before and for a period beginning 10 ms after the end of the stimulating flash. Note the change in scale of the aequorin luminescence compared to *A* and *B*. Aequorin luminescence was counted into 20-ms bins. The horizontal arrow to the right of each voltage trace indicates an absolute membrane potential of −45 mV. All traces were recorded from the same cell.

Comparing these results with those obtained using aequorin we conclude that a rise in Ca_i causes both the immediate depolarization that follows an injection of $InsPS_3$ and the subsequent oscillatory bursts. The larger quantity of EGTA needed to abolish the immediate depolarization is consistent with the larger elevation of Ca_i during the immediate depolarization, compared to that during a burst.

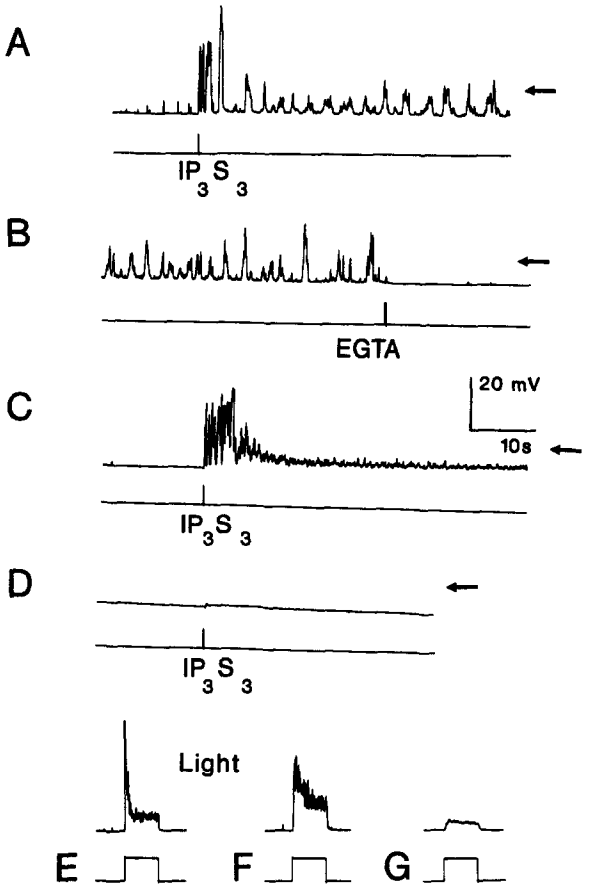

FIGURE 7. Injections of EGTA abolish the oscillations of membrane potential that follow injection of 1 mM $InsPS_3$ and greatly reduce the initial transient response to light, but do not abolish the steady, plateau response to light. (*A*) Membrane potential recorded during an injection of 1 mM $InsPS_3$. The injection elicited a series of oscillatory bursts of depolarization. (*B*) Injection of 100 mM EGTA abolished the oscillatory bursts of depolarization. This record follows and is continuous with *A*. (*C*) 100 s after the injection of EGTA shown in *B*, a subsequent injection of $InsPS_3$ produced a transient depolarization, but no sustained oscillatory bursts of depolarization. (*D*) After six further injections of 100 mM EGTA into the same cell (not shown) an injection of 1 mM $InsPS_3$ was without effect. (*E–G*) The injections of EGTA greatly reduced the initial transient response to light of the same cell used in *A–D*, but did not abolish the plateau response to light. Depolarizations caused by 5-s light flashes, log intensity −6, are shown: (*E*) before injection of either EGTA or $InsPS_3$; (*F*) after the injection of EGTA shown in *B*; (*G*) after the six further injections of EGTA delivered before the recording shown in *D*. The horizontal arrow to the right of each trace indicates an absolute membrane potential of −45 mV. The injections of $InsPS_3$ were of duration 100 ms, pressure 40 psi. The injections of EGTA were of duration 100 ms, pressure 40 psi. All traces were recorded from the same cell.

The Sensitivity to an Injection of $InsP_3$ or Light Oscillates during the Oscillatory Bursts

We investigated the effect of the $InsPS_3$-induced bursts of depolarization on the sensitivity of the photoreceptor to $InsP_3$. We impaled a cell with a double-barreled

electrode containing 1 mM $InsPS_3$ in one barrel and 100 μM $InsP_3$ in the other. Stable oscillations of membrane potential were elicited by a series of injections of $InsPS_3$ (Fig. 8*A*). For the cell of Fig. 8, the interval between the end of one spontaneous burst and the onset of the next was 13 ± 2 s (mean ± SD of seven intervals). Brief injections of $InsP_3$ were delivered to test the cell's sensitivity to $InsP_3$ at various times after a spontaneous burst (Fig. 8, *B* and *C*). The amplitudes of the depolarizations caused by the injections of $InsP_3$ were least just after a spontaneous burst of depolarization and had fully recovered before the next burst was due (Fig. 8 *C*). Oscillations of sensitivity to $InsP_3$ accompanied $InsPS_3$-induced oscillations of membrane potential in five other cells.

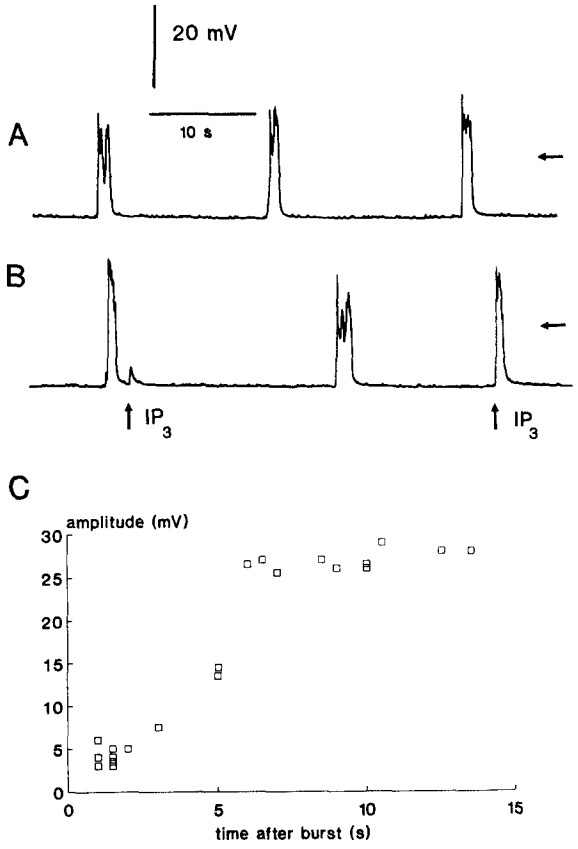

FIGURE 8. After each spontaneous $InsPS_3$-induced burst of depolarization, the sensitivity of the photoreceptor to an injection of $InsP_3$ is reduced. (*A*) Spontaneous bursts of membrane potential elicited by prior injection of 1 mM $InsPS_3$. The mean time between bursts was 13 s. (*B*) Responses to two injections of 100 μM $InsP_3$ delivered between spontaneous bursts of depolarization. The first, less effective, injection was delivered 1 s after a burst. The second injection was delivered 12.5 s after a burst. (*C*) Relationship between the amplitude of the peak depolarization caused by an injection of 100 μM $InsP_3$ and the interval between the time of occurrence of a previous burst and the time of the injection of $InsP_3$. The horizontal arrow to the right of each trace indicates an absolute membrane potential of −45 mV. All traces were recorded from the same cell.

Removal of Extracellular Calcium Slows the Frequency of Oscillations

We also investigated the role of extracellular calcium ion concentration (Ca_o) in sustaining the oscillations of membrane potential caused by $InsPS_3$. Complete replacement of Ca_o has been shown to have at least two consequences in ventral photoreceptors. First, calcium no longer enters the photoreceptor through any open

ion channels. Secondly, the mean level of Ca_i in darkness is reduced (Levy and Fein, 1985). Both of these consequences might affect the amplitude or frequency of the oscillatory bursts of depolarization.

We injected a photoreceptor with sufficient $InsPS_3$ to cause oscillations in membrane potential (Fig. 9 *A*) and then bathed the cell with 0Ca-EGTA ASW. For the cell of Fig. 9, application of 0Ca-EGTA ASW for 7 min resulted in a reduction in the mean burst frequency from 0.3 to 0.13 Hz (Fig. 9 *B*). The mean amplitude of the bursts fell only slightly from 19.7 ± 3.9 to 17.2 ± 4.2 mV. 11 min after replacement of 0Ca-EGTA ASW with ASW the burst frequency was restored to 0.3 Hz, but burst amplitude fell further to 11.6 ± 2 mV (Fig. 9 *C*). Similar reductions in the frequency of the bursts, with no significant decline in burst amplitude, were observed when three other cells were bathed in 0Ca-EGTA ASW. The results indicate that the entrance of calcium into the cytosol from the extracellular space is not necessary for producing the bursts of depolarization but that the frequency of the oscillation may be modulated either by an influx of calcium or by the mean level of Ca_i.

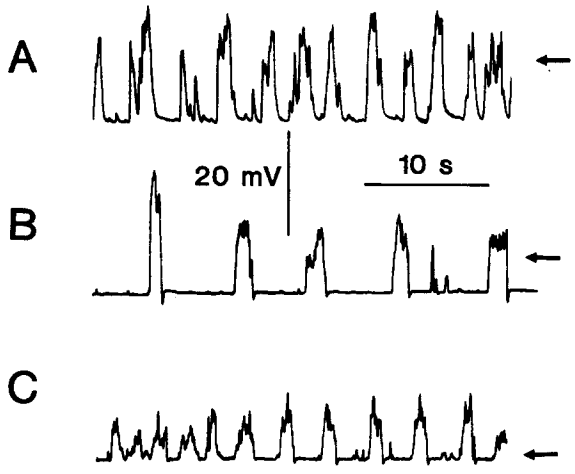

FIGURE 9. The frequency, but not the amplitude, of the $InsPS_3$-induced oscillation of membrane potential is reduced when the photoreceptors are bathed in ASW with a reduced calcium concentration. Spontaneous oscillatory bursts of depolarization were recorded after injection of 1 mM $InsPS_3$ when a photoreceptor was bathed (*A*) in ASW, (*B*) in 0Ca-EGTA ASW, (*C*) again in ASW. The horizontal arrow to the right of each trace indicates an absolute membrane potential of −45 mV.

Light Suppresses the Oscillations of Ca_i

During $InsPS_3$-induced oscillations of membrane potential, further injections of $InsPS_3$ cause a "tail" of elevated Ca_i during which oscillations of membrane potential are suppressed (Fig. 6 *B*). Steady illumination also produces a sustained elevation of Ca_i (Levy and Fein, 1985) and might therefore be expected to also suppress $InsPS_3$-induced oscillations in Ca_i and membrane potential. Cells were injected with sufficient $InsPS_3$ to induce sustained oscillations of membrane potential. Bright flashes of light reversibly suppressed the oscillations during the illumination and for a period following the cessation of illumination (Fig. 10 *B*). Dim light, which causes a lesser elevation of Ca_i also suppresses the oscillations, but only during the period of illumination (Fig. 10 *A*).

DISCUSSION

$InsPS_3$ Is an Effective Analogue of $InsP_3$ in Elevating Ca_i

$InsPS_3$ is an effective agonist of $InsP_3$ in ventral photoreceptors, as in other cells (Strupish et al., 1988; Nahorski and Potter, 1989; Taylor et al., 1989). The activity of $InsPS_3$ probably arises from the D-stereoisomer, since we find the site of action of $InsP_3$ is stereospecific. L-$InsP_3$ is much less effective than D-$InsP_3$ in depolarizing the photoreceptor. Such stereospecificity has previously been established in Swiss 3T3 cells (Strupish et al., 1988), hepatocytes (Taylor et al., 1989), and GH_3 cells (Strupish et al., 1988). Since L-$InsP_3$ was synthesized from a resolved racemic precursor, it cannot be excluded that the small depolarization observed resulted from a minor contamination with D-$InsP_3$. An identical sample was ineffective in mobilizing calcium in *Xenopus* oocytes (Taylor et al., 1988) and only weakly active in permeabilized hepatocytes (Taylor et al., 1989).

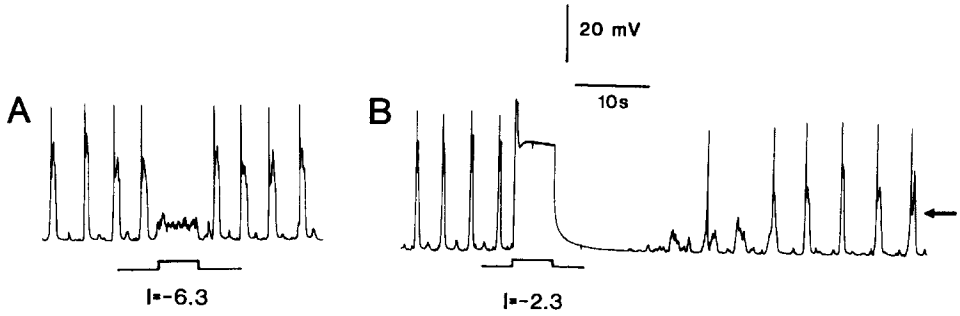

FIGURE 10. Illumination inhibits the $InsPS_3$-induced oscillations of membrane potential. Oscillations of membrane potential were recorded from a photoreceptor after the injection of $InsPS_3$. The records show the effect of illumination by a 5-s flash of intensity (*A*) −6.3 log units and (*B*) −2.3 log units. The horizontal arrow to the right of each trace indicates an absolute membrane potential of −45 mV. Both traces were recorded from the same cell.

$InsPS_3$ Is More Effective Than $InsP_3$ in Causing Prolonged Bursts of Depolarization

The relative effectiveness of an injection of $InsPS_3$ compared to that of an injection of $InsP_3$ depends on the site of the injection and the time considered after the injection. When the injection is made into the R lobe, which contains the $InsP_3$-sensitive ER, the first of a series of injections of either $InsPS_3$ or $InsP_3$ results in a similar immediate, transient depolarization. Subsequent injections, delivered a few seconds after the first, are much less effective in elevating Ca_i and so depolarizing the photoreceptor. The difference between the response to $InsPS_3$ and $InsP_3$ is evident once this period of desensitization is over. $InsPS_3$ is then much more effective than $InsP_3$ in causing bursts of depolarization that persist for many minutes. The simplest explanation for this difference is that the bursts arise from the continued presence of unmetabolized $InsPS_3$.

The known resistance of $InsPS_3$ not only to 5-phosphatase activity but also to 3-kinase activity (Nahorski and Potter, 1989; Taylor et al., 1989) makes it unlikely

that Ins(1,3,4,5)P_4 or InsP(1,4)P_2 play an important role in the phenomena demonstrated in this paper. $InsP_3$, on the other hand, would be expected to be metabolized to Ins(1,4)P_2, which is much less effective in releasing calcium or depolarizing ventral photoreceptors (Brown et al., 1984; Fein et al., 1984) and possibly also to Ins(1,3,4,5)P_4.

When the injections of $InsPS_3$ and $InsP_3$ are made into the A lobe, at a site that is distant from the R lobe, $InsPS_3$ is more effective than $InsP_3$ in depolarizing the photoreceptor, even during the initial part of the response. There is a delay of several seconds before bursts of depolarization caused by $InsPS_3$ or $InsP_3$ begin, presumably the time taken for diffusion from the A to the R lobe (Fein et al., 1984). This result suggests that in addition to limiting the time for which it acts, metabolism of $InsP_3$ confines its action within the cell.

Because of the dependence of the effectiveness of $InsP_3$ on the position of the micropipette in the cell and the high concentration of the analogues present in the micropipette, it is difficult to estimate the relative potency of $InsP_3$ and $InsPS_3$ in releasing calcium once they reach their site of action in the R lobe. The best estimate might be to compare the immediate depolarizations produced by $InsP_3$ and $InsPS_3$ when injected directly into the R lobe. At this early time, the effects of hydrolysis and dilution into the cytosol would be lessened. The similar amplitude of the immediate depolarizations produced by 1 mM $InsP_3$ and $InsPS_3$ suggests that $InsP_3$ and $InsPS_3$ might have a similar potency at the release site. In preliminary experiments, however, in which we reduced the concentration of the analogues in the injection micropipette, we have observed that 100 μM $InsPS_3$ was less effective than 100 μM $InsP_3$ in producing an immediate depolarization (unpublished observations). The similar responses to 1 mM $InsPS_3$ or $InsP_3$ might therefore be due either to local saturation of the receptor sites for $InsP_3$ or to the rapid effect of calcium-mediated negative feedback. It is likely, therefore, that the sustained effectiveness of DL-$InsP_3$ in causing bursts of depolarization is due to its resistance to hydrolysis rather than any greater potency compared to that of $InsP_3$ at its site of action.

The Mechanism of Desensitization to $InsPS_3$

Although hydrolysis of $InsP_3$ may limit its action in the long term, preventing prolonged bursts of depolarization, there is clearly another mechanism that terminates the immediate, transient depolarization that follows an injection. This mechanism must account for the rapid decline of the immediate depolarization resulting from injections of $InsPS_3$. The decline is most likely due to a reduced ability of $InsPS_3$ to release calcium, since the immediate depolarization is followed by a period of desensitization during which further injections of $InsPS_3$ are much less effective than the first. As the sensitivity returns after an injection of $InsPS_3$, bursts of depolarization begin. The short duration of these oscillatory bursts of depolarization caused by $InsPS_3$ also suggests a desensitization process. We show that the sensitivity of the cell to $InsP_3$ is suppressed after each burst of depolarization, recovering in the interval between the bursts.

Two general mechanisms might account for the period of desensitization that follows injection of $InsP_3$ or $InsPS_3$. The first is a feedback inhibition by elevated Ca_i of the ability of $InsPS_3$ to release more calcium (Ogden et al., 1990; Parker and

Ivorra, 1990; Payne et al., 1990). The second is a depletion of calcium stores following release of calcium by the first injection (Berridge, 1989). We think it possible that the first injection of $InsPS_3$ may locally deplete calcium stores in ventral photoreceptors but we cannot assess the extent of this depletion. We have, however, investigated the possibility of feedback inhibition by calcium. We have shown that an injection of calcium prior to an injection of $InsPS_3$ inhibits the ability of $InsPS_3$ to depolarize the photoreceptor. We have also shown that Ca_i remains elevated during the period of desensitization that follows a series of injections of $InsPS_3$. After this lingering elevation of Ca_i has declined, bursts of depolarization begin. Thus a feedback loop exists whereby elevated Ca_i inhibits calcium release by $InsPS_3$.

The Mechanism of the Oscillatory Bursts

Oscillations of Ca_i or Ca_i-dependent ion currents have been observed in several types of cells injected or dialyzed with $InsP_3$ or $InsPS_3$ (Oron et al., 1985; Evans and Marty, 1986; Capiod et al., 1987; Taylor et al., 1988; Wakui et al., 1989). In *Limulus* photoreceptors, the oscillations continue for at least tens of minutes following injection of $InsPS_3$. They are also occasionally observed following injections of $InsP_3$ (Corson and Fein, 1987; Payne et al., 1988), but they do not then persist for more than a few tens of seconds. The slowing of the oscillation frequency when extracellular calcium is removed, without a reduction in burst amplitude, suggests that entrance of calcium from the extracellular space plays a modulatory role in the timing of the bursts of depolarization but is not critical for the production of the bursts, in agreement with previous observations on isolated bursts induced by $InsP_3$ (Corson and Fein, 1987). In this respect the bursts are similar to those observed in some other cell types (Harootunian et al., 1988; Jacob et al., 1988; Kawanishi et al., 1989; Kurtz and Penner, 1989).

During the $InsPS_3$-induced oscillations of Ca_i we find a periodic variation in the sensitivity of the photoreceptors to $InsP_3$. Sensitivity is minimal just after a burst of calcium release and recovers in the interburst interval. It seems likely that the sensitivity to the constant presence of $InsPS_3$ might vary similarly and play a role in creating the oscillations of Ca_i. The variation in sensitivity might be due either to feedback inhibition by calcium released during the previous burst or to a variation of the amount of calcium in the $InsP_3$-sensitive ER. We think it unlikely, however, that feedback inhibition of calcium release is the sole cause of the oscillations. The reduction of burst frequency upon removal of extracellular calcium is not readily explainable by a model in which calcium only modulates the action of $InsP_3$ by negative feedback. It seems more likely that feedback inhibition is one component of a mechanism which might also include a periodic production of $InsP_3$ (Woods et al., 1987; Jacob et al., 1988; Meyer and Stryer, 1988) or calcium-induced calcium release (Berridge and Irvine, 1989; Goldbeter et al., 1990). Our result contrasts with the lack of any periodic changes in sensitivity to $InsP_3$ during oscillations of Ca_i in depolarized fibroblasts (Harootunian et al., 1988). The relative contributions of variations in the concentration of $InsP_3$ and variations in the sensitivity to $InsP_3$ to the mechanism responsible for oscillation may vary between cell types.

We also note that the peaks of aequorin luminescence observed during the oscillatory bursts of calcium release are very much smaller than the peak Ca_i reached

after a bright light flash or an injection of $InsPS_3$. Only a small fraction of the $InsP_3$- and light-sensitive store of calcium may, therefore, be released during each burst. Further work may determine whether the small fraction released is due to a spatial localization of the stores involved in creating the oscillatory bursts.

Elevation of the mean Ca_i by an exogenous stimulus such as a flash of light or an injection of $InsPS_3$ is accompanied by a subsequent desensitization of the cell and a suppression of the oscillations. Thus after a series of injections of $InsPS_3$, oscillations only begin once Ca_i has returned close to its original level, as judged by aequorin luminescence. These results suggest that the oscillations of Ca_i and membrane potential occur only when the mean Ca_i is below a certain value.

The Action of $InsPS_3$ Does Not Mimic That of Steady Illumination

Light increases the production of $InsP_3$ in *Limulus* ventral photoreceptors (Brown et al., 1984). If $InsP_3$ is the only messenger of phototransduction in *Limulus* photoreceptors, then injection of $InsPS_3$ should mimic steady illumination. This is clearly not the case. Steady illumination does not cause oscillations of Ca_i or of membrane potential, but rather a steady elevation of Ca_i (Levy and Fein, 1985) and a maintained depolarization during which oscillations of Ca_i induced by $InsPS_3$ are suppressed (Fig. 10). Our results do not support the proposal that the production of $InsP_3$ alone can account for excitation by light. This conclusion is subject to several caveats. We assume that $InsP_3$ released by light acts primarily at the same site as does injected $InsPS_3$ and that the calcium channel in the ER responds to both with similar kinetics. In addition, light delivers $InsP_3$ to the cytosol in a very different manner from the micropipette that delivers $InsPS_3$, since production of $InsP_3$ by steady light might be localized to the site of each effectively absorbed photon. Other evidence, however, supports the conclusion. The intracellular injection of the calcium chelator EGTA slows and diminishes, but does not abolish the response to light (Lisman and Brown, 1975) even though it abolishes the response to injections of $InsPS_3$ (Fig. 7) and $InsP_3$ (Rubin and Brown, 1985; Payne et al., 1986*b*). Recent work (Frank and Fein, 1990) shows that the injection of calcium chelators diminishes the amplitude of the initial transient response to a prolonged light response far more than it diminishes that of the sustained plateau response (see also Fig. 7). Thus it is possible that $InsP_3$ is involved only in generating the initial transient response and that another messenger might mediate the plateau response and suppress oscillations of Ca_i (Frank and Fein, 1990). This messenger might be other products of phosphoinositide metabolism such as diacylglycerol, metabolites of $InsP_3$, or messengers produced by other pathways, such as cyclic guanosine monophosphate (Johnson et al., 1986; Bacigalupo et al., 1990). Alternatively, $InsP_3$-induced calcium release might accelerate the production of a messenger which opens the light-sensitive channels (Bolsover and Brown, 1985).

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