A Lingering Elevation of Ca_i Accompanies Inhibition of Inositol 1,4,5 Trisphosphate–induced Ca Release in *Limulus* Ventral Photoreceptors

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ABSTRACT Injection of inositol 1,4,5 trisphosphate ($InsP_3$) into *Limulus* ventral photoreceptors causes an elevation of intracellular free Ca concentration (Ca_i) and depolarizes the photoreceptors. When measured with the photoprotein aequorin, the $InsP_3$-induced Ca_i increase follows the time course of depolarization and declines within 1–2 s. However, sensitivity to further injections of $InsP_3$ remains suppressed for several tens of seconds. The possibility that the suppression of Ca release (feedback inhibition) is due to a small lingering elevation of Ca_i, below the existing detection limit of aequorin, was investigated by measuring Ca_i with Ca-sensitive electrodes. Double-barreled, Ca-selective microelectrodes were used to pressure inject $InsP_3$ and measure Ca_i at the same point. Light or $InsP_3$ injections into the light-sensitive compartment depolarized the photoreceptors and induced an elevation of Ca_i that persisted for tens of seconds. Injections of $InsP_3$ during the decay of Ca_i showed that sensitivity to $InsP_3$ recovered as resting Ca_i approached the prestimulus level. The relationship between elevated Ca_i and feedback inhibition was very steep. An elevation of Ca_i of 1 μM or more was associated with inhibitions of 79 ± 12.4% (SEM; $n = 7$) for the $InsP_3$-induced Ca_i increase and of 76 ± 8% for depolarizations. With a residual Ca_i elevation of 0.01 μM or less, the mean inhibition was 10 ± 7.4% for $InsP_3$-induced Ca_i increase and 6.6 ± 4% for $InsP_3$-induced depolarization. Injections of $InsP_3$ into a light-*in*sensitive compartment within the cell induced elevations of Ca_i with no associated depolarizations or feedback inhibition. To verify that a sustained elevation of Ca_i is necessary for inhibition of $InsP_3$-induced Ca_i increase and depolarization, we injected ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid (EGTA) between two injections of $InsP_3$. Injection of 1 mM EGTA or the related Ca chelator BAPTA, delivered 750 ms after the first injection of $InsP_3$, restored the peak depolarization caused by the second injection of $InsP_3$ to > 80 ± 3% of control, compared with 13 ± 8% without an intervening injection of EGTA. Measurement of Ca_i with aequorin showed that an intervening injection of EGTA partially restored the $InsP_3$-induced Ca_i increase.

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The results suggest that feedback inhibition of $InsP_3$-induced Ca_i increase and depolarization is mediated by a lingering elevation of Ca_i and not by depletion of intracellular Ca stores.

INTRODUCTION

Inositol 1,4,5 trisphosphate ($InsP_3$) is thought to mediate the release of Ca from intracellular stores that occurs when *Limulus* photoreceptors are illuminated (Brown, Rubin, Ghalayini, Tarver, Irvine, Berridge, and Anderson, 1984; Fein, Payne, Corson, Berridge, and Irvine, 1984). Because of their large size, $InsP_3$ can be directly injected into *Limulus* photoreceptors. The response to a single, brief injection of $InsP_3$ comprises a rapid, transient rise in Ca_i and a consequent depolarization (Brown and Rubin, 1984; Payne, Corson, Fein, and Berridge, 1986*b*). Ca directly or indirectly initiates the opening of sodium-permeable ion channels in the plasma membrane (Payne, Corson, and Fein, 1986*a*). This response to $InsP_3$ is followed by a period of desensitization, lasting many seconds, during which the ability of a second injection of $InsP_3$ to release Ca is suppressed.

The desensitization that follows an injection of $InsP_3$ has been attributed to feedback inhibition of $InsP_3$-induced Ca release by released Ca ions (Payne, Walz, Levy, and Fein, 1988). Direct injection of Ca ions into the photoreceptor therefore also causes desensitization to subsequent injections of $InsP_3$ (Payne, Flores, and Fein, 1990). Although elevated Ca_i appears able to contribute to desensitization, the role of other factors, such as depletion of intracellular stores, is unclear. Also unclear is whether desensitization requires a sustained elevation of Ca_i that lingers after the first injection of $InsP_3$ or Ca. Alternatively, the transient elevation of Ca_i that immediately follows an injection could trigger an inhibitory mechanism that does not require continued elevation of Ca_i to sustain its action. A Ca-induced phosphorylation of the $InsP_3$ receptor might, for example, inhibit Ca release (Supattapone, Danoff, Theibert, Joseph, Steiner, and Snyder, 1988). In this article we describe experiments that address these alternative mechanisms of desensitization.

Measurement of $InsP_3$-induced Ca_i elevations in *Limulus* photoreceptors using the photoprotein aequorin as a luminescent Ca indicator (Shimomura, Johnson, and Saiga, 1962) have not detected a lingering elevation of Ca_i during the 10–20-s period of desensitization that follows an injection of $InsP_3$ (Payne et al., 1990). Detectable aequorin luminescence was limited to the 1–2 s immediately after an injection of $InsP_3$ (Payne et al., 1986*b*, 1990). These studies may have failed to make optimal use of aequorin. When used optimally, aequorin can detect resting levels of Ca_i within dark-adapted ventral photoreceptors (Bolsover and Brown, 1985; O'Day and Gray-Keller, 1989). However, the low resting aequorin luminescence under these conditions, the nonlinear relationship between Ca_i and luminescence, and the localized nature of the $InsP_3$-induced increase of Ca_i would make it difficult to quantify any elevation of Ca_i remaining after an injection of $InsP_3$, even if lingering aequorin luminescence were detectable by an improved method. We have therefore measured $InsP_3$-induced elevations in Ca_i using Ca-selective electrodes, which have a higher sensitivity to small elevations of Ca_i (Levy and Fein, 1985). After an injection of $InsP_3$ we measured an elevation of Ca_i that lingered for tens of seconds. The gradual decline of this lingering elevation of Ca_i correlated with the return of sensitivity to

subsequent injections of $InsP_3$. To test further the role of elevated Ca_i in sustaining desensitization, we also attempted to lower Ca_i transiently during the period of desensitization by injecting small amounts of the Ca chelators EGTA and BAPTA. We show that sensitivity to $InsP_3$ was restored by these injections. Taken together, these results indicate that desensitization is mediated by a sustained elevation of Ca_i acting on a rapidly reversible mechanism.

Some of the results have been reported previously in abstract form (Levy and Payne, 1991; Payne, 1991).

MATERIALS AND METHODS

Experimental Procedures

Ventral nerves from *Limulus* were pinned into a plexiglas chamber and superfused with artificial seawater (ASW) as described previously (Millecchia and Mauro, 1969; Levy and Fein, 1985).

For measurements with Ca-selective electrodes, cells were stimulated with white light from a 45-W tungsten lamp that was brought through a shutter into the specimen plane using an optic fiber (3 mm diameter). The intensity of the unattenuated white light arriving onto the photoreceptors was 45 mW/cm^2, as measured with a calibrated photometer (United Detector Technology, Santa Monica, CA). Rapid injections were achieved by applying brief pressure pulses to the injection barrel of the micropipette using a programmable pulse generator (Ionoptix Instruments, Milton, MA) and an electropneumatic valve (Clippard, Cincinnati, OH). The output of the pulse generator was connected to the chart recorder and was used to monitor the timing of the pressure pulse (stimulus monitor). Before cells were impaled, the ability of each electrode to inject was tested by ejecting solution into an oil droplet. For the injection pressures and durations used in this study, ~1 pl of solution was typically ejected. This was used to estimate a volume of 1–10 pl injected into the cell, according to the method of Corson and Fein (1983). This represents a small percentage of the cell's volume of ~400 pl (Calman and Chamberlain, 1982) and a 40–400-fold dilution once the injected material has dispersed within the cytoplasm. Experiments in which aequorin was used to monitor Ca_i were performed using apparatus described in Payne et al. (1990). Experiments were carried out at room temperature.

Ca-selective Microelectrodes

The method for measuring intracellular Ca^{2+} using Ca-selective microelectrodes is the same as that described earlier (Levy and Fein, 1985), except that double-barreled, Ca-selective microelectrodes were used. Double-barreled, Ca-selective microelectrodes were pulled from Θ glass capillaries (style 1A; R & D Glass Co., Spencerville, MD) and then slightly beveled. Photoreceptors were impaled with a voltage electrode and a double-barreled, Ca-selective microelectrode. Injections of $InsP_3$ were made through one barrel of the double-barreled microelectrode, while the resulting increase in Ca_i was measured using the adjacent Ca-selective barrel (Levy, 1992). The potential measured by the separate voltage electrode was continuously subtracted from that measured by the Ca-sensitive electrode to yield a potential (Ca signal) directly related to Ca^{2+} concentration. The calibration of Ca-sensitive electrodes and the composition of calibrating solutions have been described elsewhere (Levy and Tillotson, 1988). Although Ca-sensitive electrodes measure activities, the values are expressed as intracellular free ion concentrations.

Speed of Response of Ca-selective Microelectrodes

It was important to determine whether the double-barreled, Ca-selective microelectrodes used were fast enough to follow the decay of Ca_i after injection of $InsP_3$ or photostimulation. To measure the response time of the Ca electrode, we used a fast solution-change system described elsewhere (Coles and Tsacopoulos, 1979). We stepped the Ca concentration from 10 to 1 μM and measured the response time before and after using the Ca electrode intracellularly. Fig. 1 shows a light-induced Ca signal superimposed upon the normalized response of the Ca electrode to a step change of Ca^{2+} from 10 to 1 μM. It is clear that the decay of the Ca signal after a light flash is much slower than the response of the electrode to a step decrease in Ca^{2+}. The Ca electrode response is faster when measured before (trace *1*) rather than after intracellular measurement (trace *2;* measured 1 h after trace *1*), a typical finding for all Ca electrodes used. The time taken for the solution to change at the tip of the Ca electrode is

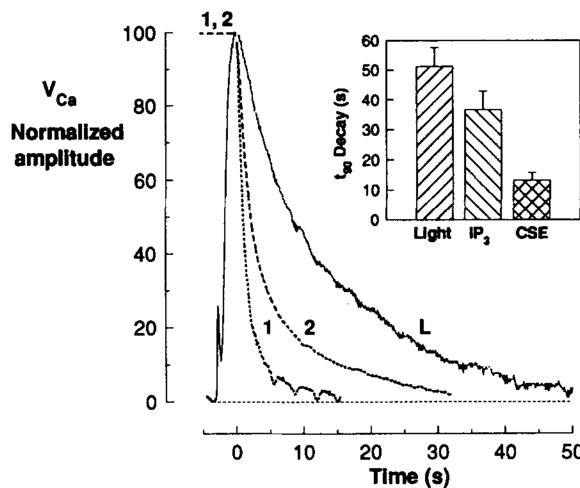

FIGURE 1. The decay of Ca_i, after an increase induced by light or $InsP_3$ injection, is not limited by the response time of the Ca-sensitive electrode. Top trace (*L*), Ca_i increase induced by a 100-ms light flash (intensity 8.8 mW/cm^2). Traces *1* and *2*, Ca electrode response to a change in Ca^{2+} concentration from 10 μM (*horizontal trace*) to 1 μM. The response time traces were measured in a fast flow system before (*1*) and after (*2*) the light response. The inset shows the average decay time (t_{90}) for the Ca_i increase induced by light or $InsP_3$ compared with the response time of the Ca electrodes (*CSE*) used between 10 and 1 μM. Student's *t* tests showed that the Ca electrode response time was significantly faster than the decay of the light-induced Ca_i increase ($P = 0.001$) and the $InsP_3$-induced Ca_i increase ($P = 0.014$). Values in the inset are means (five to eight replicates); error bars indicate SEM.

estimated to be ~25 ms (Coles and Tsacopoulos, 1979). The inset shows the average time for 90% decay (t_{90}) of the Ca signal after a light flash, an injection of $InsP_3$, or a step of Ca from 10 to 1 μM. The response of the electrodes was, on average, four times faster than the decay of the light-induced Ca signal and three times faster than the decay of the $InsP_3$-induced Ca signal.

As a further assurance that the slow response of the electrodes did not greatly distort the measurement of the decay of Ca_i transients, we compared Ca_i transients recorded using single- and double-barreled, Ca-sensitive electrodes. Because their sensing area is generally larger for the same tip diameter, single-barreled Ca electrodes are usually faster than double-barreled electrodes (Levy and Fein, 1985). We measured the $InsP_3$- and the light-induced Ca_i increase in two cells using single-barreled Ca electrodes and found their decay time to be in the same range as those measured with double-barreled, Ca-selective microelectrodes. The t_{90} for response time of the two single-barreled electrodes was < 1 s. For these experiments, $InsP_3$ was

pressure-injected from a voltage electrode tip positioned near a single-barreled, Ca-sensitive electrode.

Injection Solutions

1,4,5 $InsP_3$ was obtained either from Calbiochem Corp. (La Jolla, CA; Li salt) or Research Biochemicals Inc. (Natick, MA; K salt). Both sources yielded indistinguishable results. BAPTA [1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'* tetraacetic acid, Na salt] was obtained from Calbiochem Corp. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). EGTA was neutralized with KOH. Chemicals injected into the cells were first dissolved in carrier solution (100 mM K-aspartate, 10 mM HEPES, pH 7.20). For experiments using double-barreled, Ca-selective microelectrodes, 100 μM EGTA was added to the solution containing $InsP_3$ to prevent contamination with Ca. In all experiments $InsP_3$ was injected at a concentration of 100 μM. ASW contained (mM): 435 NaCl, 10 $CaCl_2$, 10 KCl, 20 $MgCl_2$, 25 $MgSO_4$, and 10 mM HEPES (pH 7.0). Recombinant aequorin was dissolved at a concentration of 6.7 mg/ml in carrier solution and microinjected as described earlier (Payne et al., 1990). 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 (Dept. of Chemistry, Harvard University, Cambridge, MA). Recombinant aequorin was made by incubating recombinant apoaequorin (Inouye, Noguchi, Sakaki, Takagi, Miyata, Iwagana, Miyata, and Tsuji, 1985; Inouye, Aoyama, Miyata, Tsuji, and Sakaki, 1989) with coelenterazine (Kishi, Tanino, and Goto, 1972; Musicki, Kishi, and Shimomura, 1986).

RESULTS

An Elevated Ca_i Accompanies Inhibition of Responses to $InsP_3$

Limulus ventral photoreceptors have two distinct functional lobes (Calman and Chamberlain, 1982; Stern, Chinn, Bacigalupo, and Lisman, 1982), a rhabdomeral lobe (R-lobe) which is light sensitive, and an arharbdomeral lobe (A-lobe) which is light insensitive. The A- and R-lobes can easily be identified if one strips the cells of their glia (Stern et al., 1982) or scans the photoreceptors with a microspot of light while recording the membrane potential (Levy and Fein, 1985). In previous studies it was found that light and $InsP_3$ release Ca^{2+} predominantly from the R-lobe (Levy and Fein, 1985; Payne and Fein, 1987). Instead of stripping the cells or using a microspot, we deduced that our double-barreled, Ca-sensitive electrodes were placed in the R-lobe by two criteria: first, observation of a large, fast transient elevation of Ca_i after a bright light flash (Levy and Fein, 1985); and second, a rapid depolarization after injections of $InsP_3$ (Fein et al., 1984).

Fig. 2 shows a recording of changes of both Ca_i and membrane potential after an injection of $InsP_3$ in the R-lobe. A second injection of $InsP_3$ was delivered 10 s after the first injection, while Ca_i was still elevated (Fig. 2, *left*). This injection caused a smaller depolarization and increase in Ca_i than the first one. The $InsP_3$-induced Ca_i increase was markedly inhibited when $InsP_3$ injections were spaced by 3 s (Fig. 2, *right*). Injections delivered 62 and 200 s after the first injection, after return of Ca_i to its level before the first injection, demonstrated a recovery of the ability of Ca_i to depolarize the photoreceptor and to elevate Ca_i (Fig. 2, *left* and *right*). To determine the correlation between the magnitude of inhibition and the decay of elevated Ca_i, we gave paired injections of $InsP_3$ separated by varying time intervals, corresponding to

different elevations of Ca_i after the first injection. Fig. 3 shows that the responses to the second injection of $InsP_3$ get larger as the time separating the two $InsP_3$ injections gets longer and as the level of Ca_i before the second injection gets lower. The recovery of sensitivity to $InsP_3$ seems to consist of a rapid phase that lasts ~20 s and a slower phase that lasts >100 s. The rapid phase of recovery accompanies a rapid decay of the level of Ca_i toward its value before the first injection.

The graph of Fig. 3 illustrates two details of the recovery process also observed in recordings from other cells. First, for a given injection the observed $InsP_3$-induced Ca_i increase was not necessarily inhibited to the same extent as the $InsP_3$-induced depolarization. In nine trials using six different cells, six trials showed an inhibition of the $InsP_3$-induced Ca_i increase that was greater and three had an inhibition that

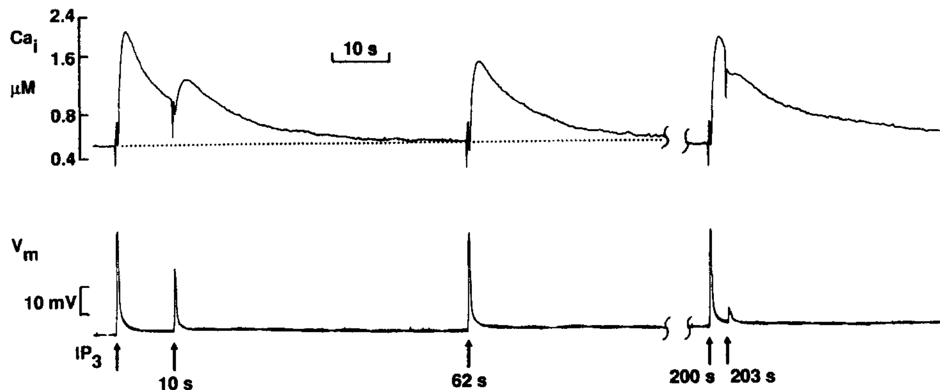

FIGURE 2. Recovery of sensitivity to $InsP_3$, at different times and at different levels of Ca_i, after the first injection of $InsP_3$ in the light-sensitive compartment (R-lobe). The $InsP_3$-induced Ca_i increase (*top trace*) and membrane depolarization (*bottom trace*) were inhibited after 10 s and recovered partially after 62 s as the level of Ca_i recovered to its preinjection level. After 200 s, the recovery was almost complete (*right*). The right panel also shows that the $InsP_3$-induced Ca_i increase was markedly inhibited when $InsP_3$ injections were spaced by 3 s. All $InsP_3$ injections: 40 PSI for 200 ms.

was less than the $InsP_3$-induced depolarization. Second, after an initially rapid phase, a slower recovery continued even after Ca_i had returned to baseline.

Inhibition of the response to $InsP_3$ can also be induced by elevation of Ca_i due to a light flash (Brown et al., 1984; Fein et al., 1984). Fig. 4 shows that after a bright flash there is a large and rapid increase in Ca_i which accompanies desensitization of the response to $InsP_3$ injections. The Ca_i levels reached by light stimulation were higher than those caused by $InsP_3$ injection and the response to $InsP_3$ after a light flash was generally more inhibited. For example, the response to $InsP_3$ was more inhibited 10 s after the light flash (Fig. 4) than 10 s after an injection of $InsP_3$ (Fig. 2), while Ca_i at the time of injection was greater (1.45 vs. 0.96 µM).

Fig. 5 shows plots of the percent inhibition of responses to a test injection of $InsP_3$ versus the elevation of Ca_i remaining after a prior injection of $InsP_3$ or a prior light flash, displaying data from all of the cells that we examined. Despite the variability

between cells, significant trends in the data can be observed. When grouped together and averaged, the eight measurements for which Ca_i was elevated by 1 μM or more before the test injection displayed 83 ± 11% (SEM) inhibition of $InsP_3$-induced Ca_i increase and 76 ± 7% inhibition of $InsP_3$-induced depolarization. By contrast, the seven measurements having a residual Ca_i elevation of 0.01 μM or less displayed a 10 ± 7.4% inhibition of the $InsP_3$-induced Ca_i increase and a 6.6 ± 4% inhibition of the $InsP_3$-induced depolarization. The average resting Ca_i in this data set was 2.29 ± 2.89 μM. (This resting Ca_i, although apparently high, is similar to that measured in a previous study using single-barreled, Ca-selective microelectrodes [Levy and Fein, 1985], but higher than the aequorin estimates of O'Day and Gray-Keller [1989]. The reasons for such an apparently high resting Ca_i are discussed extensively in Levy and Fein [1985], the most plausible one being a possible local membrane leakage around the Ca-sensitive electrode.) Third-order polynomial regression curves were drawn through the two data sets. The similarity of the two regression curves in Fig. 5 indicates that although individual cells may show variation, the $InsP_3$-induced

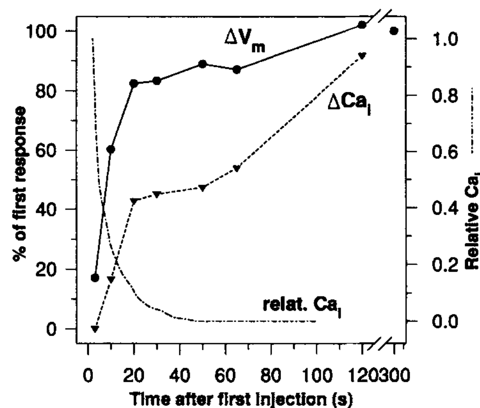

FIGURE 3. Correlation between lingering Ca_i increase and sensitivity to $InsP_3$ in the R-lobe. Plots of percent peak responses (*left axis*) of depolarization (ΔV_m, ●) and Ca_i increase (ΔCa_i, ▼) versus the time that has elapsed after the first injection of $InsP_3$. The relative Ca_i plot (*right axis*) shows the decay of Ca_i from its peak after a standard $InsP_3$ injection (40 PSI for 200 ms).

depolarization and Ca_i increase are, on average, inhibited to approximately the same extent at any given level of Ca_i. 50% inhibition is associated with elevations of Ca_i of 0.65 μM for the $InsP_3$-induced depolarization and 0.5 μM for $InsP_3$-induced Ca_i increase.

$InsP_3$-induced Elevations of Ca_i Were Also Observed After Injections into the A-Lobe

In the course of our experiments, we impaled seven photoreceptors in regions in which uniform illumination of the cell produced only a small, slow elevation of Ca_i, even though the light-induced depolarization was maximal (Fig. 6, *right;* refer to Fig. 4). Although we did not strip cells of glia to unequivocally determine the site of impalement, this weak response is typical of impalement in the A-lobe (Levy and Fein, 1985). As expected from previous work (Fein et al., 1984) in which the A-lobe was unequivocally identified, injections of $InsP_3$ into these cells elicited little or no depolarization (Fig. 6, *left*). However, we were surprised to detect, using our double-barreled, Ca-selective microelectrodes, $InsP_3$-induced Ca signals of amplitude

0.4–5.7 μM in all seven cells (Fig. 6, *left*). A previous study, using aequorin and an image intensifier to measure Ca_i, demonstrated detectable $InsP_3$-induced aequorin luminescence (Ca signal) only when injections were made into the R-lobe of the photoreceptor (Payne and Fein, 1987). The inability of aequorin to measure $InsP_3$-induced Ca_i increases in the A-lobe may be the result of its insensitivity to small elevations of Ca_i (Blinks, Wier, Hess, and Prendergast, 1982; see Discussion).

Ca signals after $InsP_3$ injections into the A-lobe had two characteristics that distinguished them from similar injections in the R-lobe: (*a*) There was no evidence of feedback inhibition. Paired injections, when spaced closely enough, led to summation of the Ca signal (Fig. 6, *right*). This result is similar to *Aplysia* neurons (Levy, 1992). (*b*) The $InsP_3$-induced increase in Ca_i decayed twice as fast to baseline as it did after injections into the R-lobe.

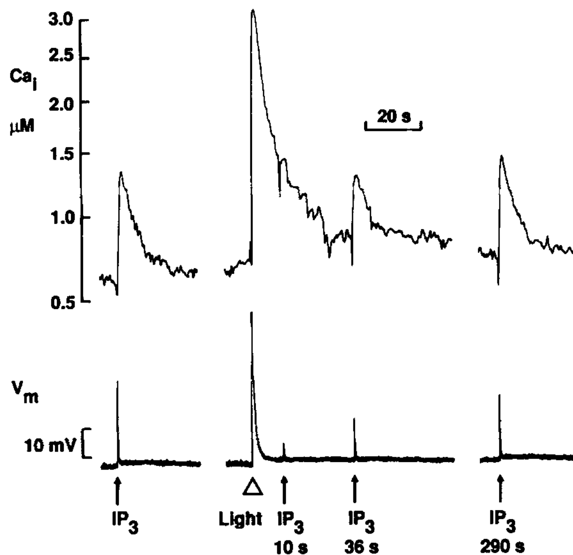

FIGURE 4. Prolonged elevation of Ca_i and desensitization to $InsP_3$ caused by light stimulation in the R-lobe. (*Left*) Control $InsP_3$ injection. (*Middle*) A 200-ms duration flash of light (intensity 8.8 mW/cm^2) caused a large rise in Ca_i and depolarization; an injection of $InsP_3$ delivered 10 s later produced no increase in Ca_i and a small depolarization. Sensitivity to $InsP_3$ started to recover 36 s after the light flash and was almost complete 290 s after the light flash (*right*) as Ca_i had recovered. All $InsP_3$ injections: 50 PSI for 200 ms.

Injection of EGTA or BAPTA Reverses Desensitization of $InsP_3$-induced Ca Release

The above experiments, using Ca-sensitive microelectrodes, indicate that a sustained elevation of Ca_i in the range 0–4 μM persists for tens of seconds after the electrical response to $InsP_3$. The rapid phase of the recovery of sensitivity to $InsP_3$ appears to accompany the decline of this lingering elevation of Ca_i. To determine whether a sustained elevation of Ca_i is necessary for inhibition of $InsP_3$-induced Ca release and depolarization, we decided to investigate the consequence of transiently lowering Ca_i by injecting small quantities of EGTA into the R-lobe. Injected EGTA will lower Ca_i at the injection site by two mechanisms. First, Ca^{2+} will be bound by EGTA. Second, because dissociation of Ca^{2+} from EGTA occurs relatively slowly (Smith, Liesegang, Berger, Czerlinsky, and Podolsky, 1984) and diffusion of Ca^{2+} is normally restricted

within the cytosol, diffusion of CaEGTA away from the injection site will accelerate the dissipation of the Ca load.

A potential pitfall is that, in addition to chelating lingering Ca_i, EGTA will also buffer, to some extent, the elevation of Ca_i produced by subsequent injections of $InsP_3$. This buffering might mask the relief of feedback inhibition by injections of EGTA. High concentrations of EGTA have clearly been shown to antagonize the response to $InsP_3$ (Rubin and Brown, 1985; Payne et al., 1986*b*). In the experiments described below, a second injection of $InsP_3$ was delivered 750 ms after the injection

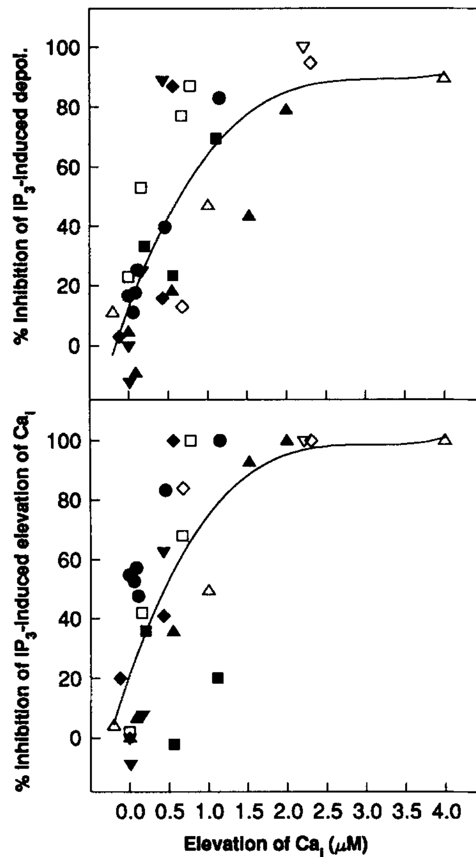

FIGURE 5. Plots of inhibition of $InsP_3$-induced depolarization (*top*) and $InsP_3$-induced Ca_i increase (*bottom*) versus the elevation of Ca_i resulting from an $InsP_3$ injection or a flash of light. Same experimental procedure as in Figs. 2 and 4. The regression lines (third-order polynomial) had an r^2 of 0.79 for the top plot and 0.74 for the bottom plot. *Filled symbols*, desensitization caused by prior injection of $InsP_3$; *open symbols*, desensitization caused by a flash of light.

of a solution containing 1 mM EGTA. In this time interval, we expect diffusion of EGTA and CaEGTA to have substantially lowered not only the total calcium load but also the concentration of EGTA at the injection site. If the injection initially created a 10-μm (4-pl) spherical bolus of 1 mM EGTA within the cell, then diffusion would be expected to reduce the combined concentration of EGTA and CaEGTA at the injection site to <0.15 mM within 750 ms (Barber, 1941, equation 143), assuming a diffusion coefficient of 10^{-6} cm^2/s for both EGTA and CaEGTA. We controlled for the effect of this lingering elevation of EGTA by determining the maximum

concentration of EGTA that could be injected without diminishing the response to a single $InsP_3$ injection. One barrel of a double-barreled micropipette was filled with a solution containing 100 μM $InsP_3$, and the other was filled with a solution containing concentrations of EGTA between 1 and 100 mM. We found that injections of a solution containing 1 mM EGTA had no effect, while injections of 10 or 100 mM EGTA progressively reduced the amplitude of the depolarization resulting from a subsequent injection of $InsP_3$ delivered 750 ms later. Injection of a solution containing 10 mM EGTA reduced a typical $InsP_3$-induced depolarization to 66 ± 13% (SEM; n = 7), while a solution containing 100 mM EGTA reduced it to 19 ± 5% (n = 4). The effect of the first 1–10-pl injection of 100 mM EGTA was partially reversible, implying that dilution of the EGTA into the ~400-pl cell volume greatly

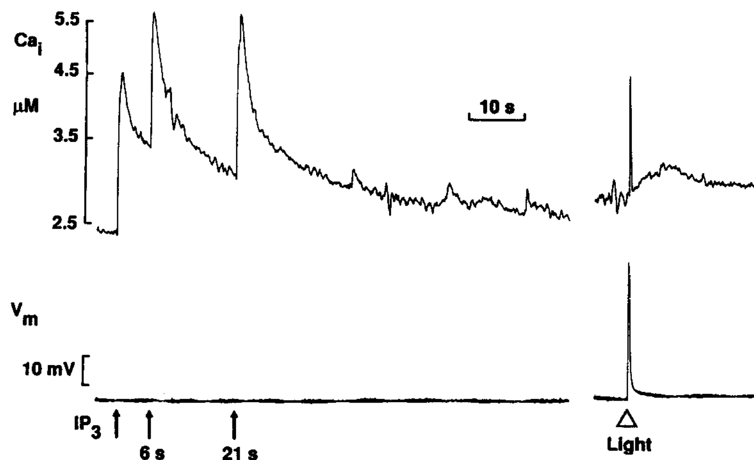

FIGURE 6. Apparent lack of feedback inhibition in the light-insensitive compartment (A-lobe). Same experimental protocol as in Fig. 2, except that the double-barreled, Ca-selective microelectrode was placed in the light-insensitive lobe. (*Left*) A first injection of $InsP_3$ does not inhibit further $InsP_3$-induced Ca_i increases. Notice the absence of depolarization. (*Right*) The light-induced Ca_i increase is small and slow, which confirms that the Ca-sensitive electrode is not in the R-lobe. There is a light-induced depolarization because the light stimulus is uniform.

reduces its effectiveness. Subsequent injections of 100 mM EGTA, however, steadily attenuated and eventually irreversibly abolished the response to $InsP_3$, as reported previously (Payne et al., 1986*b*). These experiments indicated that EGTA remaining at the injection site is saturated by the calcium released 750 ms later by a subsequent $InsP_3$ injection and is therefore unable to reduce the consequent depolarization. Injection of a solution containing 1 mM EGTA does not, therefore, prevent an $InsP_3$-induced elevation of Ca_i from occurring ~750 ms later.

The effect of injections of 1 mM EGTA on feedback inhibition was next investigated by delivering brief injections of a solution containing 1 mM EGTA between two injections of 100 μM $InsP_3$. This is shown in Fig. 7. With no intervening injection of EGTA, feedback inhibition reduced the peak depolarization caused by the second

injection of $InsP_3$ (given 1.5 s later) to 13 ± 8% of control (SEM; $n = 5$). However, Fig. 7, *D–F*, shows that injection of 1 mM EGTA, if delivered 750 ms after the first injection of $InsP_3$, partially restores the peak depolarization caused by the second injection of $InsP_3$. In a total of five cells, the depolarization induced by the second $InsP_3$ injection was restored to 80 ± 3% of control. Fig. 8 shows that, after injection of a solution containing 1 mM EGTA, sensitivity to $InsP_3$ remains elevated compared with control values for the remainder of the recovery period. However, this does not imply that EGTA necessarily remains present at the injection site throughout the recovery period. To the contrary, as noted above, we expect EGTA to rapidly diffuse from the injection site. However, the diffusion of CaEGTA along with EGTA would be

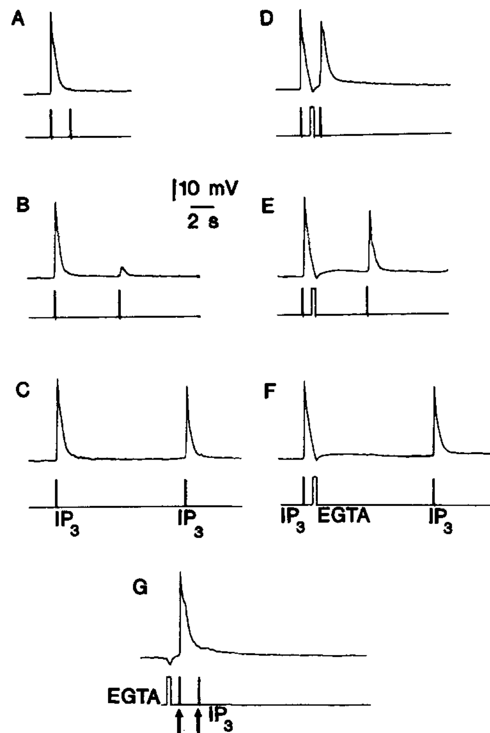

FIGURE 7. (*A–C*) Inhibition of depolarization in response to the second of two pressure injections of 100 μM $InsP_3$ delivered 1.5 s (*A*), 5 s (*B*), and 10 s (*C*) apart. (*D–F*) Reversal of inhibition produced when an injection of 1 mM EGTA is delivered 750 ms after the first injection of $InsP_3$. (*G*) Inability of injection of 1 mM EGTA to reduce either $InsP_3$-induced depolarization or subsequent inhibition when delivered 750 ms before two control injections of $InsP_3$.

expected to reduce Ca_i at the injection site for the remainder of the recovery period, accounting for the sustained recovery of sensitivity to $InsP_3$. The result of Fig. 7 *G* confirms the absence of any significant remaining concentration of EGTA shortly after the injection. Prior injection of a solution containing 1 mM EGTA has little effect either on the response to a first $InsP_3$ injection delivered 750 ms later or on the consequent inhibition of the response to a second injection 2.25 s later. Intervening injections of carrier solution between paired injections of $InsP_3$ were used as controls. In six cells, with an intervening injection of carrier solution, the depolarization caused by the second injection of $InsP_3$ was still reduced to 8 ± 3% (SEM) of control.

Intervening injections of carrier solution do not, therefore, mimic the ability of injections of 1 mM EGTA to reverse desensitization.

To control for the effects of pH changes resulting from the release of protons bound to EGTA, we substituted 1 mM BAPTA for 1 mM EGTA in the injection solution that intervened between paired injection of $InsP_3$. BAPTA is a more rapid chelator of Ca^{2+} than EGTA and one that does not release protons upon binding Ca (Tsien, 1980). The effects of an intervening injection of a solution containing 1 mM BAPTA were indistinguishable from those of EGTA. Prior injection of 1 mM BAPTA reversed desensitization of the response to the second injection of $InsP_3$ in the pair, so that the amplitude of the depolarization caused by the second injection rose from 19 ± 5% (SEM; n = 5) of that caused by the first injection to 96 ± 5%. We conclude that it is unlikely that the release of protons from EGTA upon binding Ca mediates or affects desensitization.

To verify that the effect of EGTA on the $InsP_3$-induced depolarization resulted

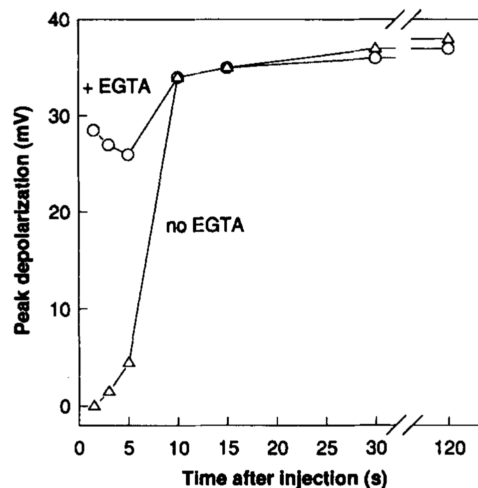

FIGURE 8. The response to the second of the paired injections of $InsP_3$ shown in Fig. 7 is plotted against the time after the first injection of $InsP_3$ either with or without an intervening injection of 1 mM EGTA.

from changes in the underlying $InsP_3$-induced Ca release, we monitored Ca_i in some cells during injections of $InsP_3$ and EGTA. We could not use the Ca electrode measurements for this purpose, since this would require a triple-barreled pipette, which would cause too much damage to the cell. Instead, Ca_i was monitored using aequorin, injected through a second single-barreled electrode. Fig. 9 *A* shows inhibition of $InsP_3$-induced elevation of Ca_i and depolarization by a prior $InsP_3$ injection, while Fig. 9 *B* shows partial recovery of both the $InsP_3$-induced depolarization and elevation of Ca_i. Results similar to those shown were obtained in three other cells. In all of these cells, the ability of EGTA injections to recover sensitivity of the second $InsP_3$-induced depolarization was greater than their ability to recover sensitivity of aequorin luminescence. A recovery of the depolarization to 74 ± 12% (SEM; n = 4) of control was accompanied by an increase of the aequorin luminescence to only 28 ± 8% of control. (Aequorin luminescence was not detectable if no EGTA injection intervened.) This discrepancy between recovery of the $InsP_3$-induced

depolarization and aequorin luminescence was noted previously (Payne et al., 1990). It could be due to the nonlinear relationship between Ca_i and luminescence and/or a saturation by excess Ca_i of the mechanism producing the depolarization.

The above experiments indicate that chelation of lingering Ca ions can rapidly reverse desensitization of $InsP_3$-induced Ca release. They also provide further evidence that desensitization results from feedback inhibition by Ca ions and not from depletion of Ca stores or control by intraluminal sites.

DISCUSSION

The results strongly suggest that feedback inhibition of Ca release by $InsP_3$ injections into the R-lobe is caused by a small lingering elevation of Ca_i. First, an elevation of Ca_i can be detected by Ca-sensitive electrodes for several seconds after an injection of $InsP_3$ or a light flash. Second, inhibition of the response to $InsP_3$ can be rapidly reversed by injection of small amounts of EGTA or BAPTA.

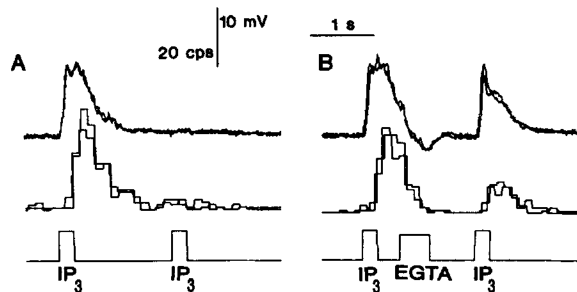

FIGURE 9. Depolarization (*upper trace*) and aequorin luminescence (*middle trace*) after paired injections of 100 μM $InsP_3$ without (*A*) and with (*B*) an intervening injection of EGTA.

Detection of $InsP_3$-induced Ca Signals in the R- and A-Lobes Using Ca-sensitive Microelectrodes

The Ca signals recorded by our Ca-sensitive electrodes after injection of $InsP_3$ differ greatly from Ca signals obtained from $InsP_3$-induced aequorin luminescence. The longer time to peak of the Ca electrode signals (0.5–2 s) compared with $InsP_3$-induced aequorin luminescence ($\sim$600 ms; Payne et al., 1986*b*) might readily be explained by the slow response of the double-barreled, Ca-sensitive electrodes (see Materials and Methods). It is likely, therefore, that the peak Ca signal reported by the electrodes greatly underestimates the true peak $InsP_3$-induced elevation of Ca_i. However, the difference between the duration of the $InsP_3$-induced Ca signal reported by the electrodes (t_{90} = 36.6 s) and that of the $InsP_3$-induced aequorin luminescence (t_{90} = 1–2 s) is not readily explainable by the electrodes' slow response (t_{90} = 13.6 s; see Materials and Methods). The electrodes therefore detect a lingering elevation of Ca_i that is undetectable by aequorin. The apparent insensitivity of aequorin, but not Ca-sensitive electrodes, to elevations of Ca_i in the low micromolar range has been previously documented for these photoreceptors with regard to the inability of aequorin to detect small, sustained elevations of Ca_i (<4 μM) associated with light adaptation (Levy and Fein, 1985). The nonlinear relationship between Ca^{2+} and aequorin luminescence is probably a major contributor (Blinks et al., 1982).

In the course of investigating Ca release in the R-lobe, we made preliminary observations of $InsP_3$-induced Ca signals into regions of cells having physiological properties characteristic of the A-lobe. In a previous study, using aequorin, $InsP_3$-induced aequorin luminescence was only observed after injections into the R-lobe (Payne and Fein, 1987). The sensitivity of the aequorin method used in that study was estimated to be of the order of 10 μM. The intensifier failed to detect spread of the light-induced elevation of Ca_i to the A-lobe, where Ca-sensitive electrodes detect increases of ~1 μM (Levy and Fein, 1985). Thus it is possible that, like the lingering elevation of Ca_i in the R-lobe, the $InsP_3$-induced Ca_i increases of 0.4–5.7 μM that we detected in the A-lobe went undetected by aequorin. Unfortunately, a detailed comparison of Ca signals recorded with Ca-sensitive electrodes in the A- and R-lobes is impractical and beyond the scope of this paper for the following reasons. The electrodes are not fast enough to detect the true peak $InsP_3$-induced elevation of Ca_i. Cell damage limits the ability to impale the same cell in both lobes and even if this were achieved, the distance between the electrode and the calcium stores might vary even within a given lobe. Our observation that the $InsP_3$-induced Ca_i increase in the A-lobe is apparently not accompanied by a subsequent desensitization to $InsP_3$ may suggest that there is more than one class of $InsP_3$ receptors. Alternatively, the $InsP_3$ receptor could be the same, but some additional factor that confers feedback inhibition could be missing in the A-lobe. The distribution of calmedin, a putative protein that may confer Ca^{2+} sensitivity to the $InsP_3$ receptor, was found to be different from that of the $InsP_3$ receptor in the brain (Danoff, Supattapone, and Snyder, 1988). The source of Ca, the shorter duration, and the apparent lack of feedback inhibition of the Ca signals in the A-lobe warrant further investigation; it would be important in particular to repeat these observations by physically verifying the exact location of the A-lobe before inserting the Ca-sensitive electrode.

Levels of Ca_i Associated with Inhibition

The levels of elevated Ca_i associated with 50% inhibition, 0.50 μM for the $InsP_3$-induced Ca^{2+} release and 0.65 μM for the $InsP_3$-induced depolarization, are consistent with previous results of 0.24–0.65 μM for inhibition of Ca release from isolated cellular membranes, permeabilized cells, and microsomes, and through $InsP_3$-sensitive ion channels (Danoff et al., 1988; Zhao and Muallem, 1990; Bezprozvanny, Watras, and Ehrlich, 1991; Finch, Turner, and Goldin, 1991). The decline of elevated Ca_i accompanies a rapid phase of recovery of sensitivity to $InsP_3$. A slow phase of recovery, accounting for ~10–20% of desensitization, may be dependent on other factors.

The level of Ca_i associated with inhibition of the response to $InsP_3$ is also comparable to that associated with light adaptation, the desensitization by bright illumination of the depolarization of *Limulus* ventral photoreceptors by flashes of light (Lisman and Brown, 1975; Levy and Fein, 1985). It is interesting to note that the recovery of the sensitivity to light, which correlates with the recovery of the Ca signal, was also found to have a fast phase of 20 s and a slower phase of 60 s or more (Fein and DeVoe, 1973; Nagy and Stieve, 1983).

The decay of Ca_i, after an $InsP_3$ injection, takes ~50 s to return to baseline in the R-lobe of *Limulus* ventral photoreceptors. There are few comparable measurements

of $InsP_3$-induced Ca_i increases in intact cells. In *Xenopus* oocytes, Parker and Ivorra (1990) found a decay time of ~15 s, measured with fluo-3, which correlated well with a time of ~13 s for the recovery of sensitivity to $InsP_3$. The decay time in *Aplysia* bursting neurons is ~50 s, measured with similar double-barreled, Ca-selective microelectrodes (Levy, 1992).

A finding yet to be resolved is the variability, at a given preinjection level of Ca_i, between the recovery of the $InsP_3$-induced Ca_i increase as compared with that of the $InsP_3$-induced depolarization. An example can be seen in Fig. 3, where the depolarization has recovered >80% 60 s after a prior injection of $InsP_3$, whereas the Ca signal has only recovered by 50%. This difference may not be a consequence of the Ca_i measuring method, since it was also observed in other studies on *Limulus* photoreceptors using aequorin (Payne et al., 1990; see also Fig. 9 *B*), and in oocytes using fluo-3 as a Ca indicator (Parker and Ivorra, 1990). Since there is firm evidence that the $InsP_3$-induced elevation in Ca_i causes the depolarization (Payne et al., 1986*b*), the discrepancy might be due to spatial differences in Ca_i level, so that Ca_i near the membrane, which influences depolarizations, may be different from that measured in the bulk of the cytoplasm.

Reversal of Inhibition by Injection of EGTA or BAPTA

80% recovery from desensitization can be rapidly achieved by injection of small amounts of EGTA (Figs. 7–9). 96% recovery from desensitization was observed after injection of 1 mM BAPTA. The incomplete recovery in some cases might be related to the 10–20% inhibition of the $InsP_3$-induced depolarization that often remained after Ca_i had naturally decayed to its baseline (for example, Fig. 3). The simplest explanation of the effect of the EGTA injections is that most of the feedback inhibition is maintained by the level of Ca_i and that there is a rapidly reversible interaction between Ca ions and the site at which inhibition is mediated. The interpretation of the results obtained using EGTA and BAPTA is subject to a caveat concerning the affinity of both agents for divalent cations like Mg^{2+} or for heavier metals than Ca. This caveat is unavoidable with nonspecific agents like EGTA or BAPTA. It may be that the effect that we observe is due to removal of a cofactor such as Mg^{2+} or zinc, which might be required by enzymes that mediate desensitization. However, our observation that injections of EGTA delivered shortly before, rather than between, paired injections of $InsP_3$ do not interfere with desensitization (Fig. 7 *G*) is difficult to reconcile with this explanation. The observation is more readily explained if we propose that EGTA removes from the injection site an ion, such as Ca^{2+}, which is released after the first injection of $InsP_3$. In other preparations, inhibition of $InsP_3$-induced Ca^{2+} release can be rapidly reversed by simply exposing permeabilized cells or microsomes to low Ca^{2+} (Zhao and Muallem, 1990; Finch et al., 1991).

Inhibition Is Not Due to Depletion of the Intracellular Ca^{2+} Store

At least two different mechanisms of Ca-mediated inhibition of $InsP_3$-induced Ca^{2+} release have been proposed. In model 1, the concentration of Ca^{2+} in the $InsP_3$-sensitive store determines the sensitivity of the Ca^{2+} release by $InsP_3$ (Nunn and Taylor, 1992). In model 2, inhibition is thought to be due to the presence of a

cytosolic factor that interacts with the $InsP_3$-sensitive channel, in a Ca^{2+}-dependent manner, to modulate inhibition of $InsP_3$-induced Ca^{2+} release (Zhao and Muallem, 1990). The factor could be an integral membrane protein (Danoff et al., 1988), a Ca-binding site on the $InsP_3$ receptor or $InsP_3$, itself produced by a Ca^{2+}-dependent phospholipase C activity (Mignery, Johnston, and Südhof, 1992).

Our results are compatible with model 2 but not with model 1. Model 1 seems to be inconsistent with several experimental findings: (*a*) Feedback inhibition can be induced by a submaximal concentration of $InsP_3$ that mobilizes part of the Ca^{2+} store (Parker and Ivorra, 1990) or by Ca^{2+} injections that do not apparently mobilize any Ca^{2+} (Parker and Ivorra, 1990; Payne et al., 1990). (*b*) Partial depletion of Ca^{2+} stores by thapsigargin has no effect on the sensitivity of $InsP_3$-induced Ca^{2+} release (Shuttleworth, 1992), in accordance with previous findings (Zhao and Muallem, 1990; Oberhuber, Maly, Überall, Hoflacher, Kiani, and Grunicke, 1991). (*c*) EGTA can rapidly reverse desensitization, as shown here.

In conclusion, our data suggest that a small lingering elevation of Ca_i in the R-lobe causes inhibition of $InsP_3$-induced Ca^{2+} release in *Limulus* ventral photoreceptors. The apparent lack of feedback inhibition in light-insensitive regions of the cell may suggest that some additional factor is also required to confer feedback inhibition.

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