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 (InsP3) 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 InsP3-induced Ca_i increase follows the time course of depolarization and declines within 1–2 s. However, sensitivity to further injections of InsP3 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 InsP3 and measure Ca_i at the same point. Light or InsP3 injections into the light-sensitive compartment depolarized the photoreceptors and induced an elevation of Ca_i that persisted for tens of seconds. Injections of InsP3 during the decay of Ca_i showed that sensitivity to InsP3 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 InsP3-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 InsP3-induced Ca_i increase and 6.6 ± 4% for InsP3-induced depolarization. Injections of InsP3 into a light-insensitive compartment 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 InsP3-induced Ca_i increase and depolarization, we injected ethyleneglycol-bis-(β-aminoethylether)-N,N'-tetraacetic acid (EGTA) between two injections of InsP3. Injection of 1 mM EGTA or the related Ca chelator BAPTA, delivered 750 ms after the first injection of InsP3, restored the peak depolarization caused by the second injection of InsP3 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 InsP3-induced Ca_i increase.

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The results suggest that feedback inhibition of InsP$_3$-induced Cai increase and depolarization is mediated by a lingering elevation of Cai 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 Cai, and a consequent depolarization (Brown and Rubin, 1984; Payne, Corson, Fein, and Berridge, 1986b). Ca directly or indirectly initiates the opening of sodium-permeable ion channels in the plasma membrane (Payne, Corson, and Fein, 1986a). 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 Cai 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 Cai that lingers after the first injection of InsP$_3$ or Ca. Alternatively, the transient elevation of Cai that immediately follows an injection could trigger an inhibitory mechanism that does not require continued elevation of Cai 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 Cai 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 Cai 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., 1986b, 1990). These studies may have failed to make optimal use of aequorin. When used optimally, aequorin can detect resting levels of Cai 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 Cai and luminescence, and the localized nature of the InsP$_3$-induced increase of Cai would make it difficult to quantify any elevation of Cai 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 Cai using Ca-selective electrodes, which have a higher sensitivity to small elevations of Cai (Levy and Fein, 1985). After an injection of InsP$_3$ we measured an elevation of Cai that lingered for tens of seconds. The gradual decline of this lingering elevation of Cai correlated with the return of sensitivity to
subsequent injections of InsP₃. To test further the role of elevated Caᵢ in sustaining desensitization, we also attempted to lower Caᵢ transiently during the period of desensitization by injecting small amounts of the Ca chelators EGTA and BAPTA. We show that sensitivity to InsP₃ was restored by these injections. Taken together, these results indicate that desensitization is mediated by a sustained elevation of Caᵢ 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/cm², 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 Caᵢ 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 Ca²⁺ 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 InsP₃ were made through one barrel of the double-barreled microelectrode, while the resulting increase in Caᵢ 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 Ca²⁺ 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 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 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 transients, we compared Ca 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 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'' 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 arhabdomeral 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 InsPs-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

![Graph](image)

**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 \( C_{ai} \) was elevated by 1 \( \mu M \) or more before the test injection displayed 83 \( \pm \) 11\% (SEM) inhibition of InsP\(_3\)-induced \( C_{ai} \) increase and 76 \( \pm \) 7\% inhibition of InsP\(_3\)-induced depolarization. By contrast, the seven measurements having a residual \( C_{ai} \) elevation of 0.01 \( \mu M \) or less displayed a 10 \( \pm \) 7.4\% inhibition of the InsP\(_3\)-induced \( C_{ai} \) increase and a 6.6 \( \pm \) 4\% inhibition of the InsP\(_3\)-induced depolarization. The average resting \( C_{ai} \) in this data set was 2.29 \( \pm \) 2.89 \( \mu M \). (This resting \( C_{ai} \), 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 \( C_{ai} \) 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 depolarization and \( C_{ai} \) increase are, on average, inhibited to approximately the same extent at any given level of \( C_{ai} \). 50\% inhibition is associated with elevations of \( C_{ai} \) of 0.65 \( \mu M \) for the InsP\(_3\)-induced depolarization and 0.5 \( \mu M \) for InsP\(_3\)-induced \( C_{ai} \) increase.

**InsP\(_3\)-induced Elevations of \( C_{ai} \) 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 \( C_{ai} \), 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 \( C_{ai} \) signals of amplitude

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**FIGURE 3.** Correlation between lingering \( C_{ai} \) increase and sensitivity to InsP\(_3\) in the R-lobe. Plots of percent peak responses (left axis) of depolarization (\( \Delta V_{m} \), ○) and \( C_{ai} \) increase (\( \Delta C_{ai} \), ▽) versus the time that has elapsed after the first injection of InsP\(_3\). The relative \( C_{ai} \) plot (right axis) shows the decay of \( C_{ai} \) from its peak after a standard InsP\(_3\) injection (40 PSI for 200 ms).
0.4–5.7 μM in all seven cells (Fig. 6, left). A previous study, using aequorin and an image intensifier to measure Caₙ, demonstrated detectable InsP₃-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 InsP₃-induced Caₙ increases in the A-lobe may be the result of its insensitivity to small elevations of Caₙ (Blinks, Wier, Hess, and Prendergast, 1982; see Discussion).

Ca signals after InsP₃ 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 InsP₃-induced increase in Caₙ decayed twice as fast to baseline as it did after injections into the R-lobe.

**Figure 4.** Prolonged elevation of Caₙ and desensitization to InsP₃ caused by light stimulation in the R-lobe. (Left) Control InsP₃ injection. (Middle) A 200-ms duration flash of light (intensity 8.8 mW/cm²) caused a large rise in Caₙ and depolarization; an injection of InsP₃ delivered 10 s later produced no increase in Caₙ and a small depolarization. Sensitivity to InsP₃ started to recover 36 s after the light flash and was almost complete 290 s after the light flash (right) as Caₙ had recovered. All InsP₃ injections: 50 PSI for 200 ms.

**Injection of EGTA or BAPTA Reverses Desensitization of InsP₃-induced Ca Release**

The above experiments, using Ca-sensitive microelectrodes, indicate that a sustained elevation of Caₙ in the range 0–4 μM persists for tens of seconds after the electrical response to InsP₃. The rapid phase of the recovery of sensitivity to InsP₃ appears to accompany the decline of this lingering elevation of Caₙ. To determine whether a sustained elevation of Caₙ is necessary for inhibition of InsP₃-induced Ca release and depolarization, we decided to investigate the consequence of transiently lowering Caₙ by injecting small quantities of EGTA into the R-lobe. Injected EGTA will lower Caₙ at the injection site by two mechanisms. First, Ca²⁺ will be bound by EGTA. Second, because dissociation of Ca²⁺ from EGTA occurs relatively slowly (Smith, Liesegang, Berger, Czerlinsky, and Podolsky, 1984) and diffusion of Ca²⁺ 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, EGTA will also buffer, to some extent, the elevation of Ca produced by subsequent injections of InsP₃. 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 InsP₃ (Rubin and Brown, 1985; Payne et al., 1986b). In the experiments described below, a second injection of InsP₃ was delivered 750 ms after the injection 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 cm²/s for both EGTA and CaEGTA. We controlled for the effect of this lingering elevation of EGTA by determining the maximum

![Figure 5. Plots of inhibition of InsP₃-induced depolarization (top) and InsP₃-induced Ca increase (bottom) versus the elevation of Caₙ resulting from an InsP₃ injection or a flash of light. Same experimental procedure as in Figs. 2 and 4. The regression lines (third-order polynomial) had an r² of 0.79 for the top plot and 0.74 for the bottom plot. Filled symbols, desensitization caused by prior injection of InsP₃; open symbols, desensitization caused by a flash of light.](image)
concentration of EGTA that could be injected without diminishing the response to a single InsP₃ injection. One barrel of a double-barreled micropipette was filled with a solution containing 100 μM InsP₃, 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 InsP₃ delivered 750 ms later. Injection of a solution containing 10 mM EGTA reduced a typical InsP₃-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 reduces its effectiveness. Subsequent injections of 100 mM EGTA, however, steadily attenuated and eventually irreversibly abolished the response to InsP₃, as reported previously (Payne et al., 1986b). These experiments indicated that EGTA remaining at the injection site is saturated by the calcium released 750 ms later by a subsequent InsP₃ injection and is therefore unable to reduce the consequent depolarization. Injection of a solution containing 1 mM EGTA does not, therefore, prevent an InsP₃-induced elevation of Ca₉ 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 InsP₃. This is shown in Fig. 7. With no intervening injection of EGTA, feedback inhibition reduced the peak depolarization caused by the second injection of InsP₃.
injection of InsP₃ (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 InsP₃, partially restores the peak depolarization caused by the second injection of InsP₃. In a total of five cells, the depolarization induced by the second InsP₃ injection was restored to 80 ± 3% of control. Fig. 8 shows that, after injection of a solution containing 1 mM EGTA, sensitivity to InsP₃ 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 expected to reduce Caᵢ at the injection site for the remainder of the recovery period, accounting for the sustained recovery of sensitivity to InsP₃. 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 InsP₃ 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 InsP₃ were used as controls. In six cells, with an intervening injection of carrier solution, the depolarization caused by the second injection of InsP₃ 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 InsP₃. BAPTA is a more rapid chelator of Ca²⁺ 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 InsP₃ in the pair, so that the amplitude of the depolarization caused by the second injection rose from 19 ± 5% (SEM; n = 5) 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 InsP₃-induced depolarization resulted from changes in the underlying InsP₃-induced Ca release, we monitored Caᵢ in some cells during injections of InsP₃ 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, Caᵢ was monitored using aequorin, injected through a second single-barreled electrode. Fig. 9A shows inhibition of InsP₃-induced elevation of Caᵢ and depolarization by a prior InsP₃ injection, while Fig. 9B shows partial recovery of both the InsP₃-induced depolarization and elevation of Caᵢ. 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 InsP₃-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 InsP₃-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](image_url)  
**FIGURE 9.** Depolarization (upper trace) and aequorin luminescence (middle trace) after paired injections of 100 nM 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 (~600 ms; Payne et al., 1986b) 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 InsP₃-induced Ca signals into regions of cells having physiological properties characteristic of the A-lobe. In a previous study, using aequorin, InsP₃-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 in 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 in the R-lobe, the InsP₃-induced Ca 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 InsP₃-induced elevation of Ca. 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 InsP₃-induced Ca increase in the A-lobe is apparently not accompanied by a subsequent desensitization to InsP₃ may suggest that there is more than one class of InsP₃ receptors. Alternatively, the InsP₃ receptor could be the same, but some additional factor that confers feedback inhibition could be missing in the A-lobe. The distribution of calmodin, a putative protein that may confer Ca²⁺ sensitivity to the InsP₃ receptor, was found to be different from that of the InsP₃ 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 Associated with Inhibition**

The levels of elevated Ca associated with 50% inhibition, 0.50 μM for the InsP₃-induced Ca²⁺ release and 0.65 μM for the InsP₃-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 InsP₃-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 accompanies a rapid phase of recovery of sensitivity to InsP₃. A slow phase of recovery, accounting for ~10–20% of desensitization, may be dependent on other factors.

The level of Ca associated with inhibition of the response to InsP₃ 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 Steve, 1983).

The decay of Ca after an InsP₃ injection, takes ~50 s to return to baseline in the R-lobe of Limulus ventral photoreceptors. There are few comparable measurements...
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of InsP₃-induced Caᵢ 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 InsP₃. 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 Caᵢ, between the recovery of the InsP₃-induced Caᵢ increase as compared with that of the InsP₃-induced depolarization. An example can be seen in Fig. 3, where the depolarization has recovered >80% 60 s after a prior injection of InsP₃, whereas the Ca signal has only recovered by 50%. This difference may not be a consequence of the Caᵢ 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 InsP₃-induced elevation in Caᵢ causes the depolarization (Payne et al., 1986b), the discrepancy might be due to spatial differences in Caᵢ level, so that Caᵢ 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 InsP₃-induced depolarization that often remained after Caᵢ 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 Caᵢ 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 Mg²⁺ 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 Mg²⁺ 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 InsP₃ 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 Ca²⁺, which is released after the first injection of InsP₃. In other preparations, inhibition of InsP₃-induced Ca²⁺ release can be rapidly reversed by simply exposing permeabilized cells or microsomes to low Ca²⁺ (Zhao and Muallem, 1990; Finch et al., 1991).

Inhibition Is Not Due to Depletion of the Intracellular Ca²⁺ Store

At least two different mechanisms of Ca-mediated inhibition of InsP₃-induced Ca²⁺ release have been proposed. In model 1, the concentration of Ca²⁺ in the InsP₃-sensitive store determines the sensitivity of the Ca²⁺ release by InsP₃ (Nunn and Taylor, 1992). In model 2, inhibition is thought to be due to the presence of a
cytosolic factor that interacts with the InsP₃-sensitive channel, in a Ca²⁺-dependent
manner, to modulate inhibition of InsP₃-induced Ca²⁺ release (Zhao and Muallem,
1990). The factor could be an integral membrane protein (Danoff et al., 1988), a
Ca-binding site on the InsP₃ receptor or InsP₃, itself produced by a Ca²⁺-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 InsP₃ that mobilizes part of the Ca²⁺ store
(Parker and Ivorra, 1990) or by Ca²⁺ injections that do not apparently mobilize any
Ca²⁺ (Parker and Ivorra, 1990; Payne et al., 1990). (b) Partial depletion of Ca²⁺ stores
by thapsigargin has no effect on the sensitivity of InsP₃-induced Ca²⁺ 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 Caᵢ in the R-lobe
causes inhibition of InsP₃-induced Ca²⁺ 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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