Facilitation of the Responses to Injections of Inositol 1,4,5-Trisphosphate Analogs in *Limulus* Ventral Photoreceptors

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ABSTRACT Injection of inositol 1,4,5-trisphosphate and its metabolically resistant analogs $InsP_3S_3$ and L-*chiro*-2,3,5-$InsP_3$ into the ventral photoreceptors of *Limulus* results in the release of calcium from internal stores and in a current flow into the cells. We show here that the dependence of the current response on the amount of analog injected is supralinear. The injections also facilitate the responses to subsequent injections. We analyze the kinetics of the responses either by very slow application of the analogs directly into the lobe that is sensitive to $InsP_3$ and light or by delivering a pulse into the nonsensitive lobe of the cell, in both cases creating a ramp of rising concentration in the sensitive region. Typically, a long latent period was followed by a strong brief inward current. The ratio between the latency and the duration of the response, defined as twice the time from half-amplitude to the peak of the response, reaches values greater than 10. Our analysis shows that this value cannot be attained within realistic models whose only nonlinearity is the cooperative binding of the ligand to its receptor. The observed ratio, however, can be achieved with a positive feedback model. Treatments that lead to partial depletion of calcium stores reversibly increase the latency of the response. We conclude that the mechanism of the response of *Limulus* ventral eye to the metabolically resistant analogs of $InsP_3$ probably involves a positive feedback mechanism and that the carrier of the feedback is likely to be Ca^{2+}.

INTRODUCTION

The phosphoinositide pathway underlies the mechanism of signal transduction in a variety of cell types. Coupling of cell-surface receptors to phospholipase C leads to an increase of $InsP_3$ concentration in the cell and subsequent release of Ca^{2+} from intracellular stores (Berridge, 1993). A single protein mediates both recognition of $InsP_3$ and transport of Ca^{2+} ions (Ferris et al., 1989).

The dynamics of Ca^{2+} release by $InsP_3$ are not yet well understood. The regulation is complex, and there is evidence that the channel is regulated by $InsP_3$ and by Ca^{2+}. Opening of Ca^{2+} channels by $InsP_3$ has been shown to be highly cooperative in rat basophilic leukemia cells (Meyer et al., 1988, 1990) and in *Xenopus* oocytes (Parker and Miledi, 1989); activation of Ca^{2+} release appears to require the binding of three to four molecules of $InsP_3$. However, in permeabilized guinea pig hepatocytes and neutrophils (Spat et al., 1986) and in the membrane vesicles from canine cerebellum (Watras et al., 1991) calcium release is linear with $InsP_3$ concentration. $InsP_3$-activated Ca^{2+} channels from frog skeletal muscle, incorporated into lipid bilayers, exhibit a linear dependence on $InsP_3$ at low Ca^{2+} levels and a cooperative dependence at high Ca^{2+} levels (Suarez-Isla et al., 1991).

A bell-shaped dependence of the $InsP_3$-induced Ca^{2+} release on the cytosolic Ca^{2+} concentration has been demonstrated in a variety of cell types (Joseph et al., 1989; Baumann and Walz, 1989; Parker and Ivorra, 1990a; Iino, 1990) and in membrane vesicles (Finch et al., 1991; Suarez-Isla et al., 1988, 1991; Bezprozvanny et al., 1991). Facilitation of $InsP_3$-induced Ca^{2+} release by the elevation of Ca^{2+} might constitute a biochemical basis for "all or none" Ca^{2+} responses with a distinct threshold. Such responses have been observed in hepatocytes (Ogden et al., 1990) and in *Xenopus* oocytes (Parker and Ivorra, 1990b).

Injections of $InsP_3$ into *Limulus* ventral photoreceptors induce transient Ca^{2+} release from the intracellular calcium stores, resulting in a transient depolarization (Fein et al., 1984; Brown et al., 1984; Brown and Rubin, 1984; Payne et al., 1986a,b; Corson and Fein, 1987). The elevation of Ca^{2+} also causes a desensitization of the response to subsequent $InsP_3$ injections and light flashes (Payne et al., 1990). The time course of the onset of this desensitization is similar to that of the declining phase of the response, suggesting that the desensitization might contribute to the transience of the response (Levitan et al., 1993). Injections of a metabolically stable analog of $InsP_3$, *myo*-inositol-1,4,5-trisphosphorothioate ($InsP_3S_3$), induce a transient response followed by oscillating bursts but no sustained response (Payne and Potter, 1991). Bursts of calcium release underlie each burst of depolarization (Corson and Fein, 1987; Payne and Potter, 1991).

To examine the mechanism of the initiation of the individual bursts in the ventral eye of *Limulus* we studied the responses to two metabolically resistant analogs of $InsP_3$, $InsP_3S_3$ (Safrany et al., 1991), and L-*chiro*-inositol-2,3,5 trisphosphate (*chr*-$InsP_3$) (Safrany et al., 1992), injected into the $InsP_3$-sensitive and $InsP_3$-insensitive lobes of the *Limulus* ventral photoreceptor. $InsP_3S_3$ is a potent $InsP_3$ 5-phosphatase inhibitor (Cooke et al., 1989; Safrany et al.,

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1991) and a Ca^{2+}-mobilizing agent with an EC_{50} (substrate concentration at which the initial reaction velocity is half maximal) some five times higher than $InsP_3$ (Safrany et al., 1991). *Chr*-$InsP_3$ is a potent Ca^{2+}-mobilizing agonist with an EC_{50} some 5 to 10 times higher than $InsP_3$, but is an inhibitor of both $InsP_3$ 5-phosphatase and 3-kinase (Safrany et al., 1992). Both analogs release Ca^{2+} in a sustained fashion in permeabilized cells.

In this study we show that after a first injection of the metabolically resistant analog into the *Limulus* photoreceptor cell there is subthreshold and suprathreshold facilitation of the response to a second injection. We show also that a slow rise of the intracellular concentration of $InsP_3$ analogs induces a fast inward current response after a long latency with a very small dispersion of the durations of latencies. We present evidence that Ca^{2+} is likely to be involved in the facilitatory mechanism.

We use modeling to explore the nature of the pathway that produces the fast inward current. We show by simulation that the combination of a long delay with a rapid rise as seen in the responses to the analogs cannot arise from cooperativity and negative feedback mechanisms alone. The addition of a positive feedback appears to be necessary and sufficient.

MATERIALS AND METHODS

The technique for preparing the ventral photoreceptors of *Limulus* was as described in detail in a preceding paper (Levitan et al., 1993). The nerves were washed and pinned into a plexiglass chamber filled with 0.5 ml of artificial sea water (ASW). In experiments that required a change of the extracellular ASW, ASW was passed through the chamber at a rate 5 ml/min; in other experiments the nerves were held in ASW without perfusion.

Intracellular recordings were performed with an Axoclamp amplifier (Axon Instruments Inc., Burlingame, CA). For current recordings, photoreceptors were clamped at their resting (dark) potentials by a conventional two-electrode voltage-clamp (Katz and Schwartz, 1974; Smith et al., 1980). An injection micropipette filled with a carrier solution containing $InsP_3$ analog was used as a voltage electrode and a micropipette filled with 2 M KCl as a current electrode. Clamp current was measured by a current-to-voltage converter in a virtual ground circuit. Current recordings were filtered by a two-pole filter with a corner frequency of 100 Hz. Under voltage clamp the cell can be considered isopotential (Brown et al., 1979).

Conventional methods for light stimulation, which were similar to those described by Fein and Charlton (1977), were used. Rapid pressure injection of substances into cells through a single-barreled micropipette was achieved as described by Corson and Fein (1983). With different electrodes different amounts of pressure had to be applied in order to inject similar amounts of the analogs. The pressure developed at the electrode holder was sensed by a miniature pressure transducer (Model PX-176–100S-5V, Omega Engineering Inc., Stamford, CT) inserted between the valve and the micropipette holder. The relative heights of the pressure recordings for different cells have no significance. We use these measurements as timing indicators and to check that the pressure during the double injection is similar to that of the single. $InsP_3$ was obtained from Calbiochem (San Diego, CA) as the trilithium salt. The stable analogs of $InsP_3$ were synthesized as described (Cooke et al., 1987; Liu et al., 1992) and were purified by ion exchange chromatography on DEAE Sephadex (Pharmacia, UK). They were used as the triethylammonium salts and quantified by the Briggs phosphate assay. Before injection $InsP_3$ and its analogs were dissolved in a carrier solution. Compositions of the carrier solution and of normal and low-Ca^{2+} ASW were the same as previously described (Corson and Fein, 1983).

The initial experiments were performed with the metabolically stable $InsP_3$ analog DL-*myo*-inositol 1,4,5 trisphosphorothioate ($InsP_3S_3$). Payne and Potter (1991) showed that at high concentrations this analog induces in the cell bursting activity that persists for tens of minutes. However, the response to the second analog of $InsP_3$, L-*chiro* 2,3,5 $InsP_3$ (*chr*-$InsP_3$), died away in *Limulus* ventral eye within 1 min. We assume that this was caused by a more rapid metabolism of the second analog, which may have arisen from its greater susceptibility to the action of nonspecific phosphatases. To avoid the gradual buildup of the analog inside the cell after repeated injections, the later experiments were performed with *chr*-$InsP_3$.

Photoreceptor cells of *Limulus* ventral eye are divided into two lobes, a light-sensitive R-lobe and a light-insensitive A-lobe (Calman and Chamberlain, 1982; Stern et al., 1982). Responses to injections of $InsP_3$ into the R-lobe are observed but not to injections into the A-lobe (Payne and Fein, 1987). Injection of the metabolically stable analogs into the R-lobe results in an immediate response, whereas injection of the analogs into the A-lobe results in a response with a considerable delay (Payne and Potter, 1991). In our experiments the R-lobes were initially identified visually as the distal part of the cell, and the identification was then confirmed by the nature of response to strong injections of $InsP_3$ or its metabolically resistant analogs. In some experiments the identification of the R-lobe was confirmed by a light spot.

The programs for numerical simulations were written in Turbo-Pascal 3, and numerical integration was performed by a Runga-Kuta method of the second degree. The parameters were optimized by minimizing the integral of the square of the difference between the experimental and calculated responses.

RESULTS

Facilitation of responses to injections of metabolically stable analogs of $InsP_3$

In our previous study we showed that an injection of $InsP_3$ into *Limulus* photoreceptor cells induces a fast desensitization of the response to a subsequent $InsP_3$ injection (Levitan et al., 1993) similar to the desensitization in *Xenopus* oocytes as described by Parker and Ivorra (1990a). However, in contrast to the observations of Parker and Ivorra we detected no facilitatory effect between injections of $InsP_3$ into the *Limulus* photoreceptor. We suggested that fast desensitization together with a short lifetime of $InsP_3$ could prevent us from seeing the facilitation. To overcome the limitation of the short lifetime of $InsP_3$ (5 s in living cells of squid (Wood et al., 1990), whereas the desensitization lasts 20–30 s), we used metabolically resistant analogs of $InsP_3$ that might be expected to have significantly longer lifetimes. With these analogs we did see a significant facilitation.

Fig. 1 *A* shows a typical pattern of responses to single and double injections of *chr*-$InsP_3$ into the R-lobe of the *Limulus* ventral photoreceptor. A single injection caused an immediate transient inward current when the cell was voltage-clamped to its resting potential (Fig. 1 *A*, top), whereas a pair of injections with a short interval between them caused a series of transient responses (bursts) of variable amplitude that lasted several seconds (Fig. 1 *A*, bottom). The time integral of the response to a double injection was 5 to 10 times larger than that of the response to a single injection. Responses to injections into the R-lobe of the second analog of $InsP_3$, $InsP_3S_3$, were similar to those shown in Fig. 1 *A*.

Fig. 1 *B* shows responses to single and double injections of *chr*-$InsP_3$ into the A-lobe of the photoreceptor. In these experiments single injections caused either a very small tran-

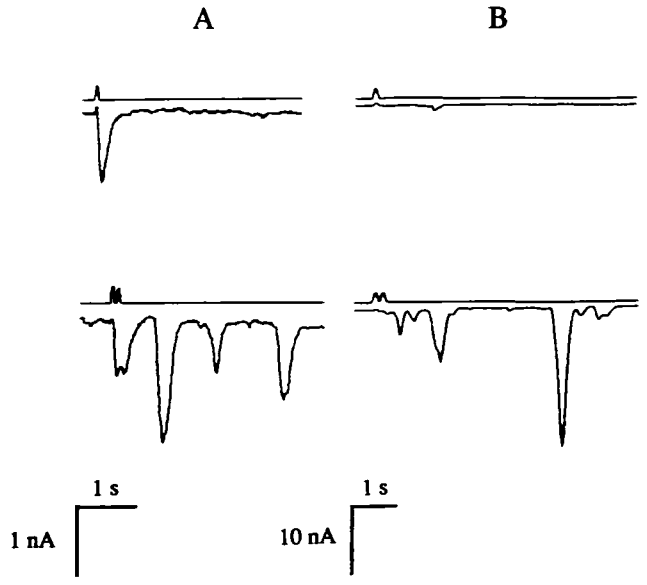

FIGURE 1 Facilitation of the responses to the injections of the *chr*-$InsP_3$. A comparison of the responses to single and double injections of 100 μM *chr*-$InsP_3$ into the R-lobe (*A*) and the A-lobe (*B*) of *Limulus* ventral photoreceptors. The cells are clamped to their resting potentials. The upper traces in the current recordings show the pressure developed at the electrode holder as sensed by a transducer. Durations of the injections were 50 ms (*A*) and 100 ms (*B*), intervals between injections 100 ms (*A*) and 200 ms (*B*). The experiment was replicated 14 times in three cells when the electrode was in the R-lobe and 12 times in three other cells when it was in the A-lobe.

sient response or none at all, whereas double injections produced large responses composed of a number of bursts starting hundreds of milliseconds after the injections. The same result was obtained with the second metabolically resistant analog $InsP_3S_3$.

Fig. 2 shows the inward current resulting from a single injection of *chr*-$InsP_3$ and from pairs of injections separated by various intervals. At intervals of 300–500 ms the time integral of the response to the second injection was five to six times larger and the amplitude four to five times larger than that to the first injection. By 1 s the facilitatory effect was down to a factor of 2.

As a measure of the facilitation, the area of the response to a double injection was divided by the area of the response to a single injection in the same cell from the same electrode. The maximum ratios varied from cell to cell. To calculate an average time course of the facilitation we normalized all the observations to their peak values. Fig. 3 shows the dependence of the normalized area ratios on the interinjection interval. The ratio at 60 s represents a control measurement of full recovery from the facilitation.

The response to the first injection into the R-lobe developed with a latency of 40–50 ms, whereas the response to the second injection was delayed by a time that depended on the interinjection interval and reached a peak of ~300 ms for an interinjection interval of 1/2 to 1 s. Fig. 4 shows the dependence of the latency of the response to the second injection on the interpulse interval.

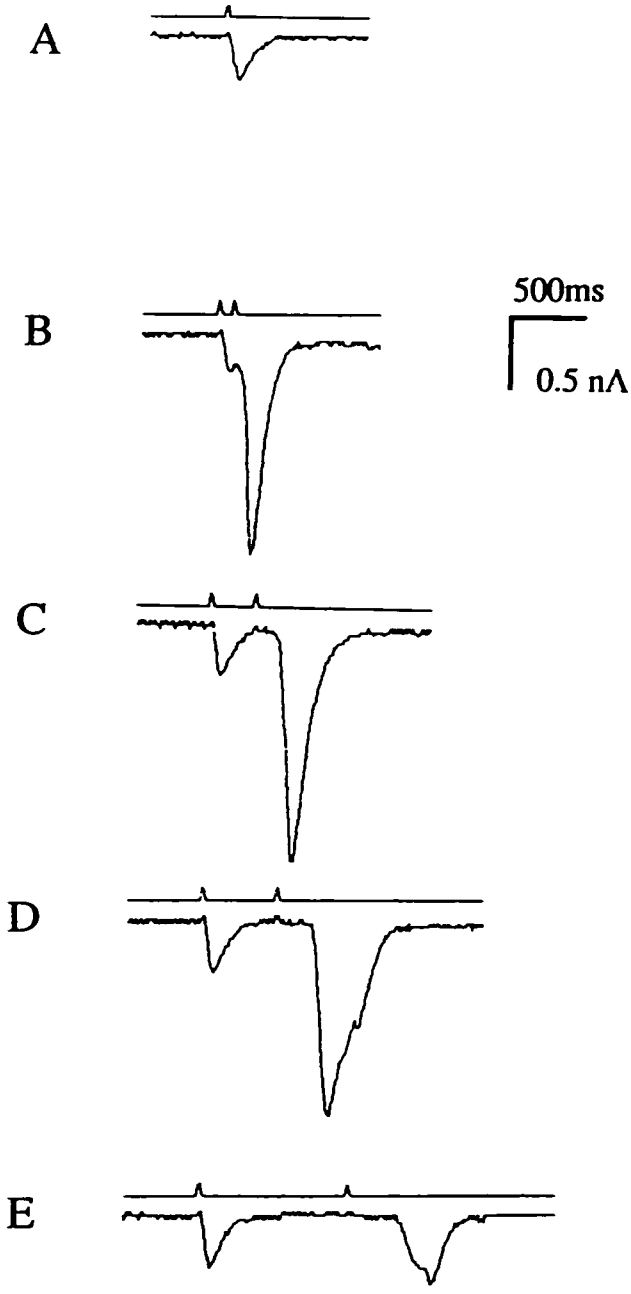

FIGURE 2 Time course of the facilitation of the responses to the injection of *chr*-$InsP_3$. Responses to single injections of 100 μM *chr*-$InsP_3$ into the R-lobe of the photoreceptor and double injections with intervals between them of 100 ms, 300 ms, 500 ms, and 1 s; injection duration 20 ms.

Further characterization of facilitation by using a slowly rising analog concentration

In this section we investigate the mechanism of the facilitation by measuring the kinetics of the response to a slow rise of the intracellular concentration of $InsP_3$ analogs. We show that a slowly rising analog concentration results in a response that grows quite suddenly after a latent period. The sharpness of this response is a measure of the degree of nonlinearity responsible for the facilitation. A concentration ramp can be

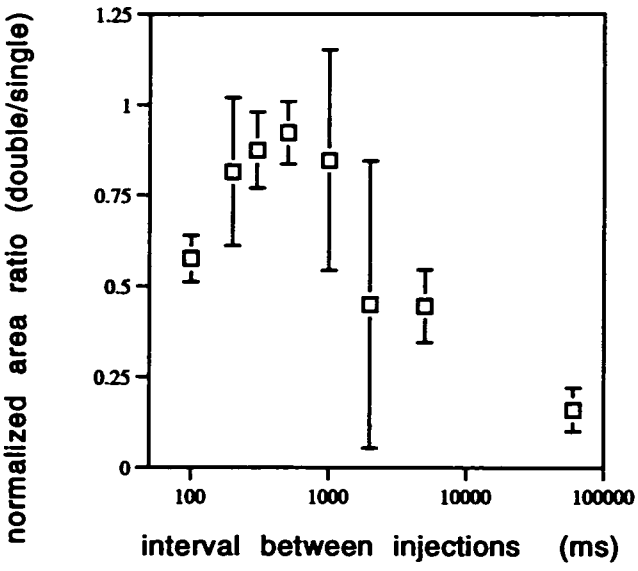

FIGURE 3 Double injection of *chr*-$InsP_3$. Dependence of the total response area on interval between injections. At short time intervals (<200 ms between injections) the responses to the two injections overlap. We therefore measured the total area of the response to the pair of injections and, as a measure of the facilitation, calculated ratios between the average area of the response to the pair of injections at a given time interval and the average area of the response to a single injection in the same cell. The points represent the averages of the normalized ratios from four different cells, two with R-lobe injection and two with A-lobe injection. The normalization was performed separately for each cell by setting the maximum ratio to 1.

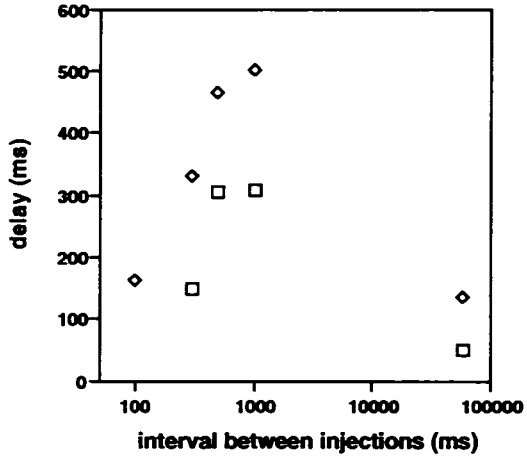

FIGURE 4 Double injection of *chr*-$InsP_3$. Dependence of the second response latency on interval between injections. The dependence of the latency of the response to the second injection of *chr*-$InsP_3$ on the interval between injections, average of two cells. Squares and diamonds represent the time from the beginning of the injection to the beginning and the peak of the second response, respectively. For the 100-ms interval the responses to the two injections overlap, so only the time to the peak could be measured.

achieved either by brief injection into the A-lobe, from which diffusion into the R-lobe should produce such a ramp, or by slow injections directly into the R-lobe.

Payne and Potter (1991) showed that injection of $InsP_3S_3$ into the A-lobe results in a response only after a considerable delay. Injections of the nonresistant $InsP_3$ into the A-lobe do not induce observable responses. We presume that $InsP_3$ is metabolized too fast to create a rising concentration ramp. Fig. 5 shows responses to injections of the second metabolically resistant $InsP_3$ analog, *chr*-$InsP_3$ into the A-lobe recorded with and without voltage clamping. The clamp kept the cells at their resting potentials. The most interesting feature of these responses is their extreme sharpness, the development of fast responses after a long silent period. In both cases the latencies of the responses, defined as the time from the beginning of the injection to the peak of the response, were about an order of magnitude longer than the durations of the responses, defined as twice the time from the rising half-amplitude to the peak of the response. The average latency/duration ratio was 8.4 ± 4.1 (four cells) without clamping the cells and 6.9 ± 2 (three cells) when the cells were clamped to their resting potentials.

We calculated the spread of response latencies (standard deviation) around the mean latency for repeated injections of the analog into the same cell by the same electrode. For every cell the experiment was performed in 10 mM Ca^{2+} ASW and also in 0 mM Ca^{2+} ASW (as will be discussed in the next section). In six cells out of seven reduction of the extracellular calcium did not affect the length of latency of the response. In these six cells the latency spread (as quantified by the standard deviation) was remarkably small, varying from 4.2 to 10.4%, with a weighted average of 8.0%.

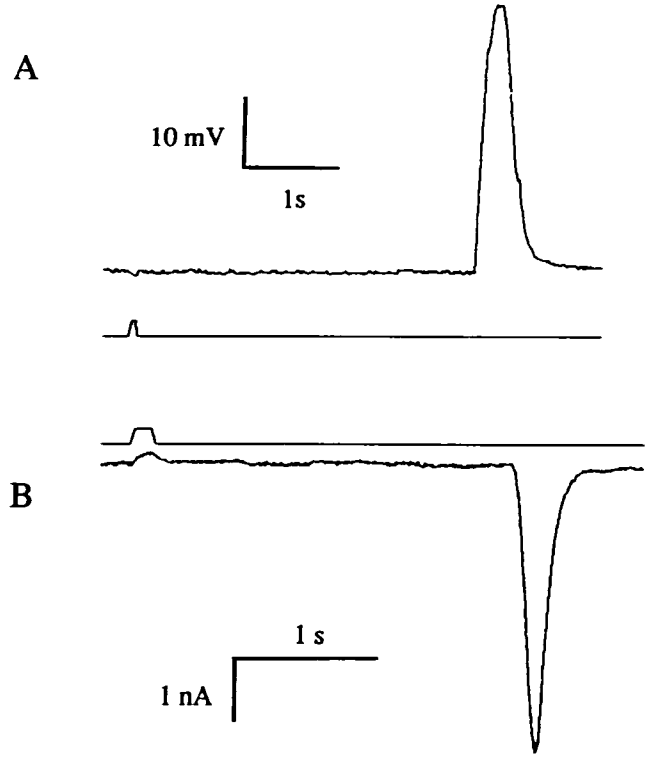

FIGURE 5 Injections of *chr*-$InsP_3$ into the A-lobe of the photoreceptor induced a fast response after long latency. Responses to the injections of 100 μM *chr*-$InsP_3$ into the A-lobe of the photoreceptor. Voltage and current recordings (clamped to resting potential).

Fig. 6 shows responses to long slow pulses of $InsP_3S_3$ injected directly into the R-lobe of the photoreceptor. A double-barreled electrode was used. One barrel contained $InsP_3S_3$ and the other $InsP_3$. To check that the location of the electrode was in the sensitive lobe, $InsP_3$ was delivered by a strong rapid (40 psi) injection; observation of a response indicated that the electrode was in the R-lobe. Injections of $InsP_3S_3$ were then delivered at low pressure (1–2 psi) for 6–8 s. Fast depolarizing responses developed several seconds after the beginning of the injection (Fig. 6 *A*) showing that long injections into the R-lobe produce a sharp onset similar to that which followed injections into the A-lobe.

$InsP_3$ injections superimposed on the steady injection of $InsP_3S_3$ were delivered by strong rapid pressure pulses (40 psi) similar to those for the control $InsP_3$ injections. In both cases $InsP_3$ injections induced immediate responses, whereas injections of $InsP_3$ delivered on the background of the long injection of $InsP_3S_3$ induced much larger responses than did the control injections (Fig. 6 *B*). These results show both facilitation of the response to $InsP_3S_3$ and sharpness of the response to $InsP_3S_3$. The average of the latency/duration ratios of the responses was 16.9 ± 1.9 (four cells). The spread of the latencies of the responses to steady injections of $InsP_3S_3$ into the R-lobe was also very small. The spread was calculated as a percentage of the mean for each cell separately, similar to the previous experiment. The spread varied from 2.5 to 13.0%, and the weighted average was 6.9%.

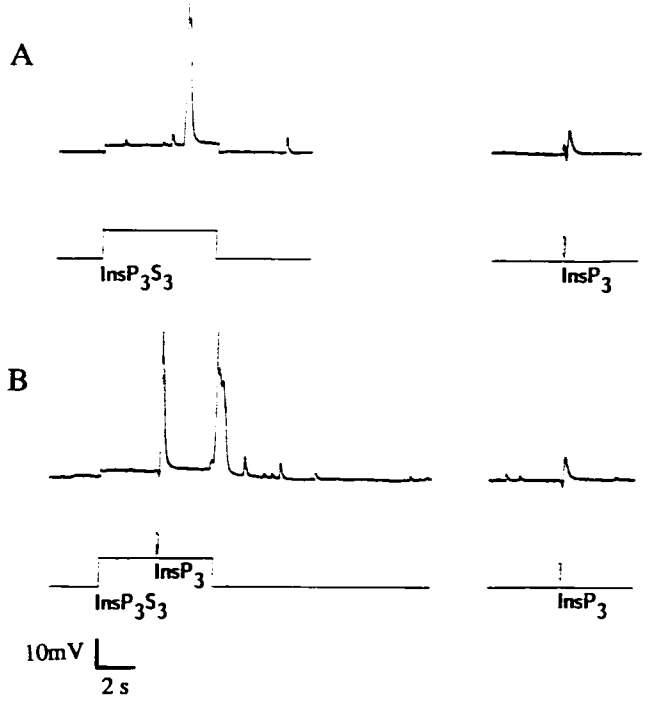

FIGURE 6 Long injections of $InsP_3S_3$ into the R-lobe of the photoreceptor induced a fast response after long latency and facilitated responses to $InsP_3$. The experiment was performed with a double-barreled electrode. One barrel contained 100 μM $InsP_3S_3$ and the second 20 μM $InsP_3$. Duration of the injection of $InsP_3S_3$ was 10 s, pressure 2 psi; duration of the injection of $InsP_3$ was 20 ms, pressure 20 psi. Injections of $InsP_3$ were given on the background of the injection of $InsP_3S_3$ and separately. The lower traces represent voltage input to the pressure-injecting device. The small random responses are presumably spontaneous "bumps," representing intrinsic activity of the photoreceptor cell.

The effect of calcium-store depletion on the latency of the response to *chr*-$InsP_3$

It has been shown in a number of preparations that Ca^{2+} induces positive feedback onto $InsP_3$-induced Ca^{2+} release (Finch et al., 1991; Bezprozvanny et al., 1991). In this section we show that Ca^{2+} may be involved in activation of positive feedback in *Limulus* ventral eye.

If calcium facilitates calcium release then the latency of the response should depend on the intracellular calcium concentration, decreasing with increasing concentration. However, the latency of the response to the injection of *chr*-$InsP_3$ into the A-lobe of the photoreceptor was not affected by lowering the resting level of calcium inside the cell (not shown). This was determined by putting the nerves in 0-Ca^{2+} EGTA ASW. The result is compatible with the observation of Payne and Flores (1992), who showed that the latency of the response to the injection of $InsP_3$ to the R-lobe was not prolonged by lowering the extracellular calcium.

However, the degree of reduction of the intracellular Ca^{2+} concentration that is produced by bathing the cell in 0-Ca^{2+} EGTA ASW is unknown and may be too small to affect the latency significantly. Reduction of the negative feedback effect of calcium may also compensate for the reduction in positive feedback when Ca^{2+} is low.

To investigate the effect of Ca^{2+} on the latency of the response to $InsP_3$ analog we decreased the rate of Ca^{2+} release by partially depleting intracellular Ca^{2+} stores. Bolsover and Brown (1985) showed that the intracellular stores can be partially depleted by exposure of the cells to flashes of bright light while the cells are immersed in 0-Ca^{2+} ASW. We used the following procedure. The nerve was incubated in normal ASW for 5–10 min, and the injection measurements were made. Then the medium was changed to 0-Ca^{2+} ASW, 15–25 brief (10 ms) flashes of bright light were delivered at 60-s intervals, and more injection measurements were taken. Typically we had to increase injection duration after the first depletion treatment because the shorter injections failed to induce electrical responses. After the injection/duration was established for the depleted state of the cell we proceeded with the same injection through the rest of the experiment. The nerve was then returned to normal ASW followed by further injection measurements. After the first recovery the depletion-recovery cycle was repeated. Fig. 7 shows responses to the injections of *chr*-$InsP_3$ into the A-lobe of the photoreceptor, before depletion (Fig. 7 *A*), after depletion (Fig. 7 *B*), and after recovery following return to 10 mM Ca^{2+} ASW (Fig. 7 *C*). Depletion causes a reversible reduction of the amplitude of the response (up to 10-fold) and an increase in latency. Table 1 shows the averages and the standard deviations of the latencies from pooled data

recorded from four cells. The measurement of the latency was repeated three to six times for each condition in every cell. The cycle of conditions (depleted-recovered) was completed twice in two cells and lacked the final "recovered" measurement in the remaining two.

MODELS

In this section we design a model, within the general framework of what is known about the inositol system, consistent with our three main observations: the facilitation of the responses to the inositol analogs by prior analog injection; the fast-rising but long-delayed response to a ramp of analog concentration, with an experimental latency/duration ratio of 6.9 ± 2 (injections of *chr*-$InsP_3$ into the A-lobe) and of 16.9 ± 1.9 (injections of $InsP_3S_3$ into the R-lobe); and the narrowness of the spread of the latencies of these responses, experimentally 8.0 ± 2.1% (A-lobe) and 6.9 ± 4.0% (R-lobe) The facilitation can be implemented by the involvement of either a cooperative step or positive feedback.

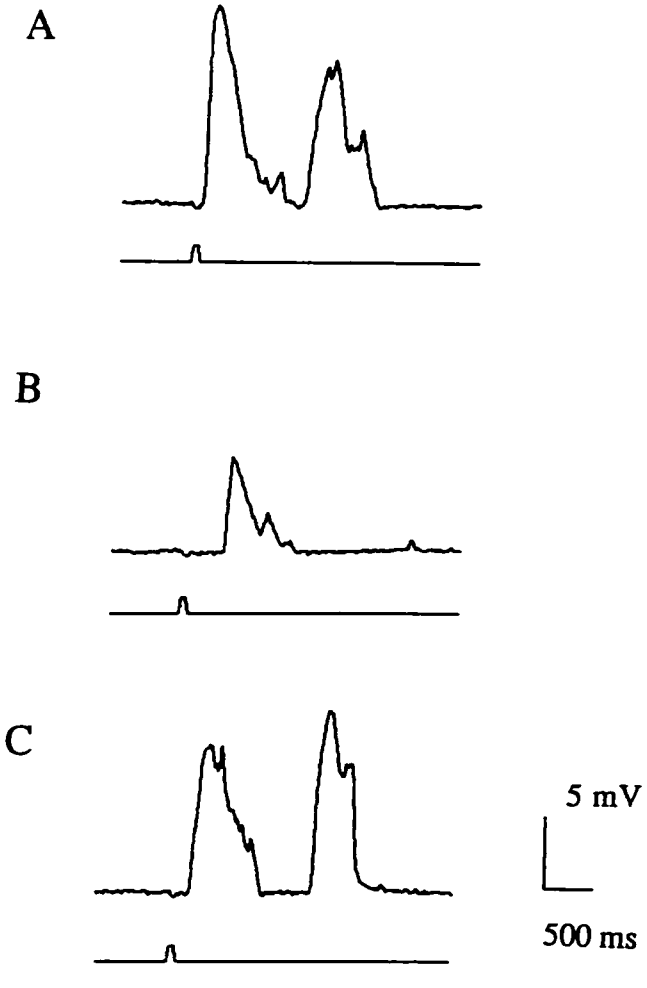

FIGURE 7 The effect of depletion of intracellular calcium stores. Responses to the injection of 100 μM *chr*-$InsP_3$ into the A-lobe of the photoreceptor before depletion (*A*), after depletion of Ca^{+2} by exposure to flashes of bright light while the cell is immersed in low-calcium ASW (*B*), and after recovery (Ca^{+2} added to ASW) (*C*).

TABLE 1 Depletion of the Intracellular Stores Increases the Length of Latency of the Responses to the Injections of *chr*-$InsP_3$

Depleted 1	Recovered 1	Depleted 2	Recovered 2
1.55 ± 0.16	1 ± 0.14	1.78 ± 0.34	0.91 ± 0.18

Measurements are ratios of latencies in the depleted states and in the second recovered state to those in the first recovered state. The variability of the measurements comes in part from the fact that a steady-state condition is not reached in this experiment. After a significant portion of the intracellular Ca^{2+} is depleted every injection causes more depletion. Latencies of the recovered states were measured after the cells were immersed in 10 mM Ca^{2+} ASW medium for 10 min.; however, the recovery process continued to some extent after this time.

One possible mechanism for achieving a high latency/duration ratio is the activation of an all-or-none response by a delayed process. Such a process could be the triggering of a massive action by the arrival of one or a few molecules at any one of many receptors. This process, however, is stochastic, and leads to a spread of "first-arrival" times. We calculate in Appendix A that for single-molecule arrivals, this spread is 52.2%, whereas if activation of the receptor requires four molecules, the spread becomes 14.8%. We measure much smaller spreads and accordingly reject this model.

In the body of this section we simulate the effects, on the latency/duration ratio, of the presence of a cooperative stage and of two successive cooperative stages. For cooperativities of 4 at each of two stages we are able to reach latency/duration ratios no larger than 2.1.

Finally, we model the effects of a positive feedback on the form of the response and find it easy to simulate the observed latency/duration ratio. We conclude that positive feedback is sufficient to explain the observations.

Simulation

A general diagram of the elements involved in the excitation chain of the response to $InsP_3$ is presented in Fig. 8. Binding of $InsP_3$, and perhaps Ca^{2+}, creates an open form of the calcium channel (R^*_{Ca}) by activation of a closed form of the channel (R_{Ca}). Elevation of the cytoplasmic calcium concentration (Ca_c) by release of calcium from the intracellular stores (Ca_s) generates a positive inward current by opening the Na^+ channels in the plasma membrane. Ca^{2+} released from the stores feeds back to desensitize and perhaps also to facilitate its release. The inset in Fig. 8 shows the hypothesized scheme of activation-desensitization of the Ca^{2+} channel. We assume that both active (R^*_{Ca}) and inactive (R_{Ca}) forms of the channel may create a desensitized (R^D_{Ca}) form of the channel. The excitation chain can be divided into five steps.

Elevation of $InsP_3$ concentration

We assume that both slow injection of the analog into the R-lobe and diffusion of the analog from a pulse injection into

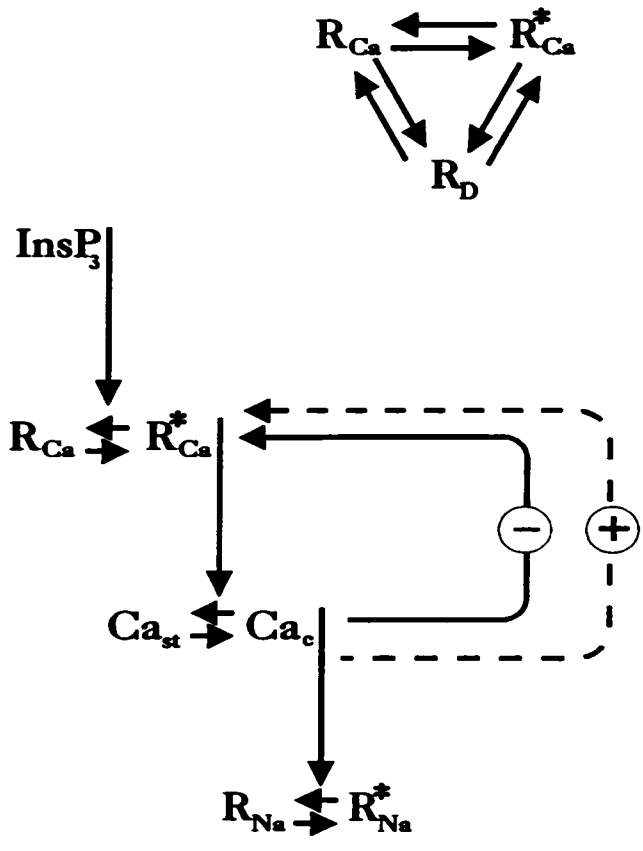

FIGURE 8 Regulation mechanisms of the $InsP_3$ pathway. Schematic representation of the intermediates of $InsP_3$ pathway with a negative feedback loop (*solid line*) and a positive feedback loop (*dashed line*) from the elevation of calcium level in the cell to the activation of the $InsP_3$-receptor. The insert of the figure shows the hypothesized scheme of the activation and the desensitization of the Ca^{2+} channel. See text for details.

the A-lobe create a slow ramp of analog concentration with the time course of a first-order Γ distribution. This distribution has approximately the same latency/duration ratio as was obtained from a simple diffusion calculation. The equation used for numerical simulation is

$$\mathrm{InsP_3}\,(t) = at\,e^{-bt}. \tag{1}$$

We have checked other forms, such as a linear rise in concentration. This is likely to be a closer approximation to reality for the direct injection into the R-lobe. We also used Γ distributions of the second and third order. None of these significantly changed the calculated response time courses.

$InsP_3$-induced opening of calcium channels

Binding of $InsP_3$, and perhaps of Ca^{2+}, creates an open form of the calcium channel (R^*_{Ca}) by activation of a closed form of the channel (R_{Ca}). Let r^*_{Ca} be the fraction of the channels that are open and r_{Ca} be the fraction of the channels that are closed but not desensitized by the negative feedback. The rate constants of $InsP_3$ binding to the receptor and of $InsP_3$ dissociation from the receptor are k_{ip3} and k_{-ip3}, respectively. The rate constants for Ca^{2+} binding and dissociation are k_{Ca} and k_{-Ca}, respectively. Assuming fast equilibrium of the intermediate stages of the receptor the rate of the receptor activation will be proportional to a power of the ligand concentration, where the power is the number of ligand molecules required to activate the receptor.

$$\mathrm{d}r^*_{Ca}/\mathrm{d}t = k_1(\mathrm{IP_3})^{n_1}(\mathrm{Ca_c})^{n_2}r_{Ca} - k_{-1}r^*_{Ca} \tag{2}$$

where n_1 is the cooperativity of $InsP_3$ in Ca^{2+}-channel opening and n_2 is the cooperativity of Ca^{2+} in activation of the positive feedback; Ca_c is the calcium concentration in the cytoplasm. k_1 has the dimensions of $s^{-1}M^{-(n_1+n_2)}$ and is the ratio of the rate constants $(k_{Ca})^{n_2}(k_{ip3})^{n_1}/(k_{-Ca})^{n_2}(k_{-ip3})^{n_1-1}$. We carried out the simulations for $n_1 = 1$, 2, and 4, and $n_2 = 0$ (no positive feedback), 1, and 2. A sample derivation of Eq. 2 is given in Appendix B.

Release of Ca^{2+} from intracellular stores

Opening of the calcium channels releases calcium from the intracellular stores (Ca_{st}) to the cytoplasm (Ca_c) with a maximal Ca^{2+} flux V_2. The rate of calcium pumping into the stores is taken as a saturable function and a maximum rate of V_p with an EC_{50} of K_p. Then

$$\frac{\mathrm{dCa_c}}{dt} = V_2\,r^*_{Ca}(\mathrm{Ca_{st}} - \mathrm{Ca_c}) - V_p\frac{\mathrm{Ca_c}}{K_p + \mathrm{Ca_c}}. \tag{3}$$

We assume that Ca_c is a small fraction of Ca_{st}, so that Ca_{st} is taken as a constant. We checked the assumption by simulating cases in which Ca_c was 15 and 30% of Ca_{st}. Such depletion of Ca_{st} had no significant effect on the shape of the time courses of the open channels. We also assume that Ca_c removal from the cytoplasm is not saturated. Saturation of this removal will cause a prolongation of the response and a decrease in the latency/duration ratio. With the assumptions that Ca_c is a small fraction of Ca_{st} and that pumping of Ca_c is not saturated, and with the substitutions $k_2 = V_2\mathrm{Ca_{st}}$ and $k_{-2} = V_p/K_p$, we obtain

$$\mathrm{d}Ca_c/\mathrm{d}t = k_2 r^*_{Ca} - k_{-2}\mathrm{Ca_c}. \tag{3a}$$

Ca^{2+}-induced opening of Na^+ channels

Elevation of the intracellular calcium (Ca_c) level generates a positive inward current by opening sodium channels (R_{Na}) in the plasma membrane. Let r_{Na} and r^*_{Na} be the fractions of channels closed and open, respectively. The description of the activation of the Na^+ channels is similar to the activation of Ca^{2+} channels.

$$\mathrm{d}r^*_{Na}/\mathrm{d}t = k_3(\mathrm{Ca_c})^{n_3}r_{Na} - k_{-3}r^*_{Na} \tag{4}$$

where n_3 is the cooperativity for Ca^{2+} activation of Na^+ channels, k_3 has dimensions of $s^{-1}M^{-n_3}$ and is the ratio of the rate constants $(k_{Ca_2})^{n_3}/(k_{-Ca_2})^{n_3-1}$, where k_{Ca_2} is the rate constant of Ca binding to the channel and k_{-Ca_2} is the rate constant for Ca dissociation. We performed simulations for $n_3 = 1$, 2, and 4.

Ca^{2+}-mediated negative feedback onto Ca^{2+} release

Termination of the response by a calcium-induced negative feedback might be caused either by competition between calcium and $InsP_3$ (or its analog) for the receptor or to inactivation of the receptor following or independently of $InsP_3$ binding. We relate here to the case in which calcium can bind both to the active (R^*_{Ca}) and to the inactive (R_{Ca}) forms of the channel, creating a desensitized R^D_{Ca} complex. We have previously shown that the time course of the negative feedback is slower than the time course of the response to $InsP_3$ injection (Levitan et al., 1993). For this reason we do not apply the fast binding approximation to the onset of the negative feedback but simulate it in n_4 sequential steps (n_4 is the cooperativity of Ca^{2+} in the negative feedback).

Let r_{Di} be the fraction of the intermediate form of the receptor when i ions of Ca^{2+} are bound to the desensitization sites; then,

$$\frac{dr_{D_i}}{dt} = k_4(Ca_c)r_{D_{i-1}} - k_{-4}r_{D_i} - k_4(Ca_c)r_{D_i} + k_{-4}r_{D_{i+1}} \quad (5)$$

where k_4 is the rate constant of Ca^{2+} binding to the desensitization sites of the receptor, k_{-4} is the rate constant of Ca^{2+} dissociation from these sites, and i is the number of the desensitization sites occupied by Ca^{2+}. r_{D_0} is the sum of the fractions of the closed and open forms of the channels that have no Ca^{2+} ions bound to their desensitization sites.

The simulations were performed for two cases, that in which occupation of one desensitization site by Ca^{2+} is sufficient to desensitize the receptor and that in which all four sites must be occupied for the desensitization. The conservation equations are:

$$\sum_{i=0}^{n_4} r_{D_i} = r_{totCa} \quad (6)$$

$$r^*_{Na} + r_{Na} = r_{totNa}. \quad (7)$$

We performed numerical simulations for various combinations of nonlinear stages of the $InsP_3$ pathway. We divided the possible combinations of the nonlinear stages into four main cases and optimized the values of the parameters for best fit with the experimental results in each case. Values of the optimized parameters for different model are given in the caption to Fig. 8. The figures show the time courses of channel opening optimized to the time course of the inward current response resulting from an injection of *chr*-$InsP_3$ into the A-lobe of the photoreceptor. The cases are:

Model A. Release of calcium requires the binding of several molecules of $InsP_3$ to each receptor molecule (n_1 = 1, 2, or 4, n_2 = 0, n_3 = 1), whereas the binding sites are independent of each other. Termination of the response is caused by a cooperative negative feedback loop (n_4 = 4). The models yield latency/duration ratios 0.56, 0.63, and 0.77, respectively, and is incompatible with the experimental results (Fig. 9 *A*). If the binding of one molecule increases or decreases the affinity of the unoccupied binding sites the conclusion is unaffected. Termination of the response by a linear negative feedback (n_4 = 1) results in slowing the falling phase of the response and decreasing latency/duration ratios (not shown).

Model B. There are two cooperative steps in series, with an $InsP_3$ cooperativity of 4 (n_1 = 4, n_2 = 0) and two or four calcium molecules required to activate sodium conductance (n_3 = 2 or 4). The response is terminated by a cooperative negative feedback loop (n_4 = 4). Two sequential steps with a cooperativity of 4 each yield an overall cooperativity of 16. Even this cooperativity is not enough to produce the observed response sharpness. Latency/duration ratios are 1.4 and 2.1 for calcium cooperativities of 2 and 4, respectively (Fig. 9 *B*).

Model C. Ca^{2+} activates a positive feedback onto $InsP_3$-induced Ca^{2+} release (n_2 = 1 or 2). Among the models for supralinearity described above, this model predicts the strongest facilitation. The number of $InsP_3$ molecules required to open a Ca channel is one or four (n_1 = 1 or 4, n_3 = 1, n_4 = 4). Cooperativity of $InsP_3$ increases the sharpness of the response, and latency/duration ratios of 2.1 and 4.9 are obtained for n_1 = 1 and 4, respectively, with n_2 = 1. If two molecules of Ca^{2+} are required in order to activate the positive feedback, the latency-duration ratio increases to 10.5 (n_1 = 1, n_2 = 2). Fig. 9 *C* shows that a positive feedback mechanism can produce responses with latency/duration ratios that are compatible with the observed latency/duration ratio.

Model D. Ca^{2+} activates a positive feedback onto $InsP_3$-induced Ca^{2+} release and both $InsP_3$ and Ca^{2+} bind cooperatively to activate the next stage. The cooperativity of $InsP_3$ is 4 and the cooperativity of Ca activation of Na channels is 2 or 4 (n_1 = 4, n_3 = 2 or 4, n_4 = 4). The latency/duration ratios are 6.5 and 9.6 for n_3 = 2 or 4, respectively. This model too is compatible with the observed sharpness of the responses (Fig. 9 *D*).

Within models that we have tested we were able to simulate all of the observed responses only within the models that include positive feedback.

DISCUSSION

Facilitation of the responses to injections of stable analogs of $InsP_3$

Injection of $InsP_3$ into *Limulus* ventral photoreceptors has been shown to facilitate the response to light flashes (Fein et al., 1984; Brown et al., 1984). In this paper we have shown that injection of the metabolically stable analogs of $InsP_3$, $InsP_3$ and *chr*-$InsP_3$ into *Limulus* ventral photoreceptors facilitates the response to subsequent injections of these analogs. Facilitation persists when the cell is clamped to its membrane potential, showing that it has a biochemical basis in the mechanism of the response to $InsP_3$ and does not result from the voltage-dependent regenerative properties of the membrane.

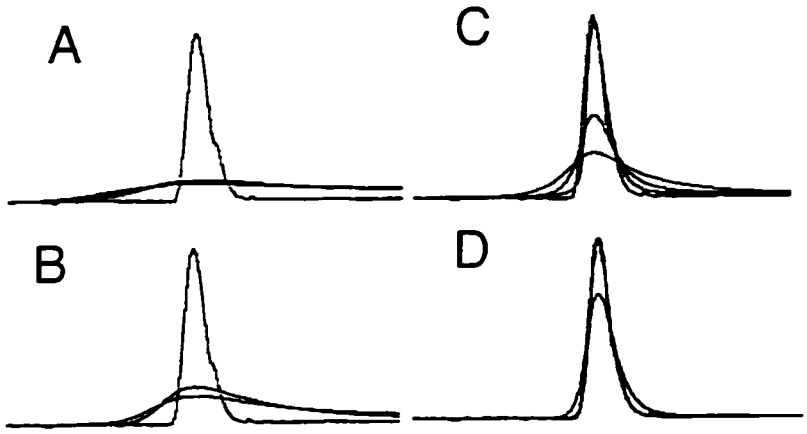

FIGURE 9 Simulations of the response to the injections of stable analogs of $InsP_3$. Comparison of the response to injection of *chr*-$InsP_3$ into the A-lobe of the photoreceptor (the same curve for A, B, C and D) and the simulated responses to a slowly rising ramp of $InsP_3$ concentration. Simulated responses show the time courses of the concentration of open sodium channels optimized to the experimental current response. We performed the simulations with a time step of 10 ms similar to the sampling rate of the recordings. We have not measured Ca^{2+} concentration in the cell; for this reason we cannot relate the optimized parameters to real concentrations of these substances but use hypothetical concentration units (u). The peak Ca^{2+} concentrations of the responses for the best fitted simulated responses (positive feedback) varied around 150 u; resting Ca^{2+} concentration was assumed to be 1 u. Injection of $InsP_3$ into *Limulus* ventral eye induces elevation of the intracellular Ca^{2+} concentration greater than 1.5–2 μM (Levy and Payne, 1993). The simulated responses are obtained by numerical integration of the following models. The cooperativities n_1–n_4 apply to processes as seen in Fig. 8, and the values given for the k parameters of equations are the result of the optimization. Where the values are not given they are as for the preceding model. For the cases in which $n_4 = 4$, the rate constant k_4 was determined by optimizing four steps of Ca^{2+} binding to four binding sites and not by a fast binding approximation as for the other rate constants. This is because we have previously shown that the time course of the onset of the negative feedback is slower than the time course of the response to $InsP_3$ injection. $InsP_3$ time course is as follows. $InsP3(t) = 0.01e^{(-0.001t)}$.

Model A: (n_1 = 1, 2, or 4; n_2 = 0; n_3 = 1, n_4 = 4): Cooperative binding of $InsP_3$ to the calcium channel. The rate constants are 1) lowest peak: $k_1 = 6.6\ u^{-1}s^{-1}$, $k_{-1} = 100\ s^{-1}$, $k_2 = 3.9\ us^{-1}$, $k_{-2} = 38.1\ s^{-1}$, $k_3 = 51\ u^{-1}s^{-1}$, $k_{-3} = 10\ s^{-1}$, $k_4 = 5.9\ u^{-1}s^{-1}$, $k_{-4} = 0.1\ s^{-1}$; 2) intermediate peak: $k_1 = 5.8\ u^{-2}s^{-1}$, $k_2 = 4.9\ us^{-1}$, $k_4 = 7.5\ u^{-1}s^{-1}$; 4) highest peak: $k_1 = 5.5\ u^{-3}s^{-1}$, $k_2 = 4.9\ us^{-1}$, $k_4 = 9.2\ u^{-1}s^{-1}$.

Model B: (n_1 = 4, n_2 = 0, n_3 = 2 or 4, n_4 = 4): Two sequential cooperative steps. The cooperativity of the first is 4; the second step has a cooperativity of 2 (lower peak: $k_1 = 4\ u^{-3}s^{-1}$, $k_3 = 11.5\ u^{-2}s^{-1}$, $k_4 = 9.2\ u^{-1}s^{-1}$) or 4 (higher peak: $k_1 = 3.1\ u^{-3}s^1$, $k_3 = 0.39\ u^{-4}s^{-1}$, $k_4 = 9.2\ u^{-1}s^{-1}$).

Model C: (n_1 = 1, 2, or 4; n_2 = 1 or 2; n_3 = 1, n_4 = 4): Positive feedback combined with a noncooperative binding of $InsP_3$ (lowest peak: $k_1 = 0.051\ u^{-2}s^{-1}$, $k_{-1} = 10\ s^{-1}$, $k_2 = 100\ us^{-1}$, $k_{-2} = 100\ s^{-1}$, $k_3 = 59\ u^{-1}s^{-1}$, $k_{-3} = 100\ s^{-1}$, $k_4 = 1.6\ u^{-1}s^{-1}$, $k_{-4} = 0.1\ s^{-1}$), a cooperative binding of 4 (intermediate peak: $k_1 = 0.032\ u^{-3}s^{-1}$, $k_3 = 60\ u^{-1}s^{-1}$, $k_4 = 1.8\ u^{-1}s^{-1}$), or cooperative Ca^{2+} activation of the receptor (n_1 = 1, n_2 = 2) (highest peak: $k_1 = 0.01\ u^{-5}s^{-1}$, $k_3 = 90\ u^{-1}s^{-1}$, $k_4 = 1.62\ u^{-1}s^{-1}$).

Model D: (n_1 = 4, n_2 = 1, n_3 = 2 or 4, n_4 = 4): Positive feedback combined with a cooperative binding of $InsP_3$ and a cooperative binding of Ca, cooperative binding of 2 (lower peak: $k_1 = 0.03\ u^{-5}s^{-1}$, $k_3 = 1.1\ u^{-2}s^{-1}$, $k_4 = 2.6\ u^{-1}s^{-1}$) or 4 (higher peak: $k_1 = 0.03\ u^{-5}s^{-1}$, $k_3 = 3.8 \cdot 10^{-4}\ u^{-5}s^{-1}$, $k_4 = 2.0\ u^{-1}s^{-1}$).

Parker and Miledi (1989) showed facilitation between injections of $InsP_3$ in *Xenopus* oocytes. We have not seen facilitation with $InsP_3$ but only with its stable analogs. The failure to observe facilitation with $InsP_3$ could result from the local nature of the $InsP_3$-induced calcium release when $InsP_3$ is pressure-injected into the cell. Injection of $InsP_3$ induces a fast desensitization that suppresses the responses to subsequent injections over times between 200 ms and a number of seconds later (Payne et al., 1990; Levitan et al., 1993). Unlike the stable analogs, $InsP_3$ may be hydrolyzed before it diffuses out of the region of the desensitization into regions where facilitation can occur.

The facilitatory effect can occur even in the absence of an observable response to the facilitating injection, as was sometimes seen in A-lobe injection. Both analogs facilitate the response to light (not shown), as does $InsP_3$ (Fein et al., 1984). $InsP_3S_3$ also facilitates the response to an injection of $InsP_3$ (Fig. 6). These interactions and the similarity between the effects of the two analogs suggest that the facilitatory effect is an integral part of the $InsP_3$ pathway.

The facilitatory effect continues for several seconds, and maximum facilitation is observed after intervals of 500 ms–1 s. The delayed peaking of facilitation may be the result of the action of a positive feedback in which facilitation is induced not by the $InsP_3$ analog itself but by one of the later intermediates of the excitation chain.

However, it is also possible that this effect may result from the spatio-temporal distribution of the analog in the cell. Injection of $InsP_3$ or an analog releases calcium locally in the region of the injection, causing desensitization of that region; a second injection could release calcium not from the desensitized region but rather from the remaining nondesensitized region, which it takes time for the injected analog to reach. Increasing the interval between the injections allows the first to diffuse to a larger area, and increases the portion of the cell that is facilitated.

Injection of a stable analog into the R-lobe not only facilitates the response to a subsequent injection but also increases its latency. We suggest that this effect results from the desensitization of the local region of the cell in the

vicinity of the injecting pipette. The material from the second injection has to bypass the desensitized volume in order to induce a response, and this takes time. The size of the desensitized volume grows with time after the first injection so that the latency of the response to the second injection increases with the interval.

Involvement of a positive feedback in the response to the $InsP_3$ analogs

It has been shown in a number of tissues that release of calcium depends on $InsP_3$ concentration in a highly nonlinear way. The mechanism of the supralinearity is either a cooperativity of $InsP_3$ binding to its receptor (Meyer et al., 1988, 1990; Parker and Miledi, 1989) or a positive feedback of Ca onto $InsP_3$-induced Ca release (Joseph et al., 1989; Iino, 1990; Finch et al., 1991; Bezprozvanny et al., 1991) or a combination of both.

To distinguish among these possibilities, we induced a steadily rising concentration of the analogs in the cell. Injections of $InsP_3$ itself under these conditions generally gave no responses, apparently because of its rapid hydrolysis in the cell.

Slow application of metabolically resistant analogs of $InsP_3$ induces fast responses after a silent period that can be an order of magnitude longer than the duration of the fast response itself. The process of diffusion to the receptor binding sites cannot produce a sharp concentration change. So a threshold mechanism appears to be required.

We showed that the high reproducibility of the latency in a given experiment makes it unlikely that a stochastic process is responsible for inducing the burst. A single molecular event like the opening of one Ca^{2+} channel cannot therefore be the mechanism responsible for the sharpness of the observed response.

Numerical simulations of cooperative binding models including models of independent binding sites and positive cooperativity between the sites and two sequential cooperative steps show that none of these can produce an apparent threshold compatible with the observed sharpness of the responses (Fig. 9). Introduction of a positive feedback mechanism increases the sharpness of the simulated responses so that their latency/duration ratio becomes similar to that of the experimental observations (Fig. 9).

Calcium as a possible intermediate of the positive feedback mechanism

The final question concerns the factor responsible for the positive feedback. Calcium has been shown to facilitate $InsP_3$-induced calcium release in other tissues (Joseph et al., 1989; Iino, 1990; Finch et al., 1991; Bezprozvanny et al, 1991). Calcium can also act as a co-agonist of $InsP_3$ in isolated $InsP_3$-sensitive calcium channels (Suarez-Isla et al., 1991; Bezprozvanny et al., 1991). However, there is little prior evidence of calcium facilitation of $InsP_3$ responses in *Limulus* ventral eye. Reduction of the resting calcium level of the cell by immersing the cells in 0-Ca^{2+} ASW extracellular medium results in an increase in the interburst interval during the oscillatory phase of the response (Payne and Potter, 1991), but it does not cause the prolongation of the latency of the response to $InsP_3$ (Payne and Flores, 1992) or to *chr*-$InsP_3$ (not shown). We suggest that the reduction of the resting Ca^{2+} by this treatment is too small to affect significantly the latency of the responses.

The observation that partial depletion of the intracellular calcium stores indeed causes a reversible prolongation of the latency (Fig. 7, Table 1) is consistent with the possibility that Ca^{2+} serves as the mediator of the positive feedback, and suggests that the absence of an effect of earlier treatments on the response latency is caused by their failure to deplete the intracellular Ca^{2+} stores sufficiently.

In conclusion, the mechanism of the response to the stable analogs of $InsP_3$ includes a highly supralinear step. To explain the sharpness and the small variability of latency in the responses to the analog injections, we have presented a model that adds a Ca^{2+}-induced positive feedback to the generally postulated Ca^{2+}-induced negative feedback. This combination of positive and negative feedbacks results in a response whose time course is much faster than that of the $InsP_3$ concentration itself.

APPENDIX A: SCATTER OF ONE-MOLECULE EVENT TIMES

In this appendix we calculate the latency spread that would arise from the induction of $InsP_3$ responses by the first arrival of single $InsP_3$ molecules at any one of an ensemble of receptors.

We may consider a large collection of independent $InsP_3$ receptor molecules, each of which eventually will be triggered by a time-varying concentration of $InsP_3$. Their independence assures that the early events will conform statistically to a time-dependent Poisson point process. The probability that the first event has not yet occurred will be of the form

$$P(t) = \exp\left(-\int_0^t \lambda(t)\,dt\right) \tag{A1}$$

which is thereby determined once the time-dependent expected event rate $\lambda(t)$ is known. Eq. A1 determines the s^{th} moment of the first-event time as

$$\langle t^s\rangle = \exp\left(-\int_0^t t^s\,dP(t)\right). \tag{A2}$$

The fractional variability of the first-event time can be naturally measured as the ratio of its standard deviation to its mean, the "coefficient of variation"

$$C = \frac{(\langle (t-\langle t\rangle)^2\rangle)^{1/2}}{\langle t\rangle} = \left(\frac{\langle t^2\rangle}{\langle t\rangle^2} - 1\right)^{1/2} \tag{A3}$$

evaluated from Eq. A2 by letting $s = 1$ and $s = 2$.

Suppose each receptor molecule has n sites for $InsP_3$ attachment and that as soon as they are filled an event occurs. Suppose the $InsP_3$ concentration rises linearly, starting at 0 when $t = 0$. We can show that up to the time of the first event (and unless we insist on perversely large ratios of rate constants, such as the total number of receptor molecules) the law of mass action will effectively describe the occupation of the first $n - 1$ sites; and further, we can show that in this circumstance, both depletion and dissociation can be ignored. Even if interactions among sites yield distinct rate constants, we can verify the result that $\lambda(t) \sim t^{2n-1}$, so that Eq. A1 takes the form

$$P(t) = e^{-\alpha t^{2n}} \tag{A4}$$

(where α is a constant involving the kinetic parameters). From this in turn Eq. A2 yields (substitute $\alpha t^{2n} = x$):

$$\langle t^s \rangle = \alpha^{-s/2n} \int_0^\infty x^{s/2n} e^{-x}\, dx = \alpha^{-s/2n}((s/2n)!) \qquad \text{(A5)}$$

where the generalized factorial $y! = \Gamma$ function $(y - 1)$ is a little more convenient than its related Γ function. On substitution into Eq. A3, the dependence on α cancels between numerator and denominator, giving

$$C(n) = \left(\frac{(1/n)!}{((1/2n)!)^2} - 1 \right)^{1/2} \qquad \text{(A6)}$$

as the coefficient-of-variation measure of the fractional variability of the first-event time. We tabulate this measure through cooperativities that run as high as $n = 4$: $n = 1$, $C(n) = 0.523$; $n = 2$, $C(n) = 0.281$; $n = 3$, $C(n) = 0.194$; $n = 4$, $C(n) = 0.148$. Coefficients of variation measured were 0.08 ± 0.021 (*chr*-$InsP_3$, six cells) and 0.069 ± 0.040 ($InsP_3S_3$, four cells). We note that other experimental sources of noise can only increase the variation. Given that the cooperativity is unlikely to be more than 4 we reject this model.

APPENDIX B

In this appendix we show the derivation of Eq. 2 for the best-fit case of model C: two ions of Ca^{2+} and one molecule of $InsP_3$ are required to activate the receptor. To simplify the derivations we assume that Ca^{2+} must bind first, and only then does the affinity to $InsP_3$ increase sufficiently to allow the binding of $InsP_3$. Alternative assumptions affect the ratios of the rate constants but not the powers of the ligand concentrations, and therefore do not alter the conclusions.

The receptor sequentially binds two ions of Ca^{2+} and then one molecule of $InsP_3$. The resulting complex forms an open state of the channel.

$$R \leftrightarrow RCa1 \leftrightarrow RCa2 \leftrightarrow RCa2I$$

The rate equations for the fractions of the receptor are:

$$\frac{d(rCa1)}{dt} = k_c(Ca)(r) - k_{-c}(rCa1) - k_c(Ca)(rCa1) + k_{-c}(rCa2) \qquad \text{(B1)}$$

$$\frac{d(rCa2)}{dt} = k_c(Ca)(rCa1) - k_{-c}(rCa2) - k_i(Ip3)(rCa2) + k_{-i}(rCa2I) \qquad \text{(B2)}$$

$$\frac{d(rCa2I)}{dt} = k_i(Ip3)(rCa2) + k_{-i}(rCa2I). \qquad \text{(B3)}$$

Optimization of the rate constants for these differential equations shows that the channel-opening time course has the largest latency/duration ratio for low Ca^{2+} and $InsP_3$ concentrations. For these concentrations the fraction of the channels that have no Ca^{2+} ion bound is much greater than the fraction with one Ca^{2+} ion bound ($r \gg rCa1$), and the fraction of the channels that have one Ca^{2+} ion bound is much greater than the fraction with two Ca^{2+} ions bound ($rCa1 \gg rCa2$). For this condition we may rewrite Eqs. B1 and B2 as follows:

$$\frac{d(rCa1)}{dt} = k_c(Ca)(r) - k_{-c}(rCa1) \qquad \text{(B4)}$$

$$\frac{d(rCa2)}{dt} = k_c(Ca)(rCa1) - k_{-c}(rCa2) \qquad \text{(B5)}$$

We assume that the binding of Ca to the activation sites of the receptor is fast so that the intermediate states rCa1 and rCa2 are in a quasi-equilibrium state. We then solve these equations to get:

$$(rCa) = \left(\frac{k_c}{k_{-c}} \right)(Ca)(r) \qquad \text{(B6)}$$

$$(rCa2) = \left(\frac{k_c}{k_{-c}} \right)(Ca)(rCa1) = \left(\frac{k_c}{k_{-c}} \right)^2 (Ca)^2(r) \qquad \text{(B7)}$$

and

$$\frac{d(rCa2I)}{dt} = k_i \left(\frac{k_c}{k_{-c}} \right)^2 (Ip3)(Ca)^2(r) - k_{-i}(rCa2I) \qquad \text{(B8)}$$

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