The Role of the Inositol Phosphate Cascade in Visual Excitation of Invertebrate Microvillar Photoreceptors

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ABSTRACT The identity of the transmitter(s) involved in visual transduction in invertebrate microvillar photoreceptors remains unresolved. In this study, the role of inositol 1,4,5-trisphosphate (IP_3) was examined in *Limulus* ventral photoreceptors by studying the effects on the light response of heparin and neomycin, agents that inhibit the production or action of IP_3. Both heparin and neomycin reduce responses to brief flashes of light and the transient component of responses to steps of light, and also inhibit IP_3-induced calcium release, indicating that IP_3 plays a direct role in invertebrate visual excitation. The effects of BAPTA, a calcium buffer, were also examined and shown to be consistent with a role for IP_3-mediated calcium release in visual excitation. However, all three agents fail to block the plateau component of the response to a step of light, indicating that a single pathway involving IP_3 and calcium cannot solely be responsible for visual excitation in invertebrates. We suggest that the inositol phosphate cascade and a second parallel process that is not dependent on IP_3 are involved in the production of the light response.

INTRODUCTION

Invertebrate microvillar photoreceptors respond to a flash of light with a membrane depolarization resulting from the opening of cation channels in the plasma membrane. The intracellular messenger produced by light that is responsible for causing these channels to open has not been definitely established. One candidate for this role is inositol 1,4,5-trisphosphate (IP_3), a water-soluble product of the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), a minor component of the plasma membrane (Grado and Ballou, 1961; Tomlinson and Ballou, 1961). Initial support for the messenger role of IP_3 came from studies which showed that injections of IP_3 into *Limulus* ventral photoreceptors produce transient membrane depolarizations which result from underlying membrane currents having the same reversal potentials as those produced by brief flashes of light (Brown et al., 1984*b*; Fein et al., 1984).

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Volume 97 April 1991 697–723

Further support comes from biochemical studies demonstrating light-induced increases in IP_3 or its degradation product, inositol 4,5-bisphosphate (IP_2) in *Limulus* ventral photoreceptors (Brown et al., 1984*b*), squid retinas (Szuts et al., 1986; Brown et al., 1987; Wood et al., 1989) and fly photoreceptors (Devary et al., 1987; Inoue et al., 1988). Finally, the *Drosophila* no receptor potential (norpA) mutant has been shown to have abnormally low levels of phospholipase C (PL-C) activity, the enzyme that cleaves PIP_2 to form IP_3 (Inoue et al., 1985; Yoshioka and Inoue, 1987), and the norpA protein (defective in the norpA mutant) has extensive sequence similarity to a known PL-C (Bloomquist et al., 1988).

Nevertheless, the extent to which IP_3 mediates excitation by light remains controversial, mainly due to the different effects of calcium chelators on IP_3-induced vs. light-induced depolarizations. While both light (Brown and Blinks, 1974; Brown et

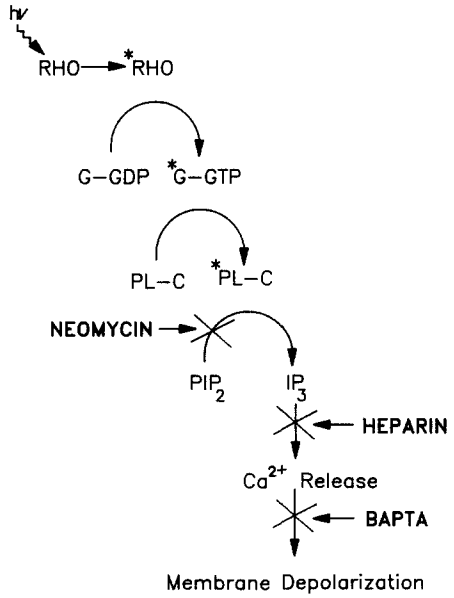

FIGURE 1. Putative cascade of visual transduction in invertebrate microvillar photoreceptors, after Fein (1986). According to this model, photoactivated rhodopsin (*RHO*) activates a GTP-binding protein (*G*), which activates phospholipase C (*PL-C*), stimulating the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) and producing inositol 1,4,5-trisphosphate (IP_3) as one of the products. IP_3 stimulates the release of calcium from intracellular stores, leading to the receptor potential. Locations where heparin, neomycin, and BAPTA would block the cascade are indicated. The asterisk signifies an activated molecule.

al., 1977; Nagy and Stieve, 1983; Bolsover and Brown, 1985; Levy and Fein, 1985) and injections of IP_3 (Brown and Rubin, 1984; Payne et al., 1986*b*; Corson and Fein, 1987) induce a release of calcium from intracellular stores, calcium buffers that suppress excitation to injected IP_3 (Rubin and Brown, 1985; Payne et al., 1986*b*) and injected calcium (Payne et al., 1986*a*), only slow excitation produced by light (Lisman and Brown, 1975*b*; Payne et al., 1986*a*, *b*). Further evidence detracting from the role of IP_3 as the messenger of visual excitation is that large or prolonged injections of IP_3 produce a series of transient depolarizations (Brown et al., 1984*b*; Fein et al., 1984; Payne et al., 1988), while a step of light (>1 s) produces a biphasic depolarizing response consisting of a transient peak followed by a steady-state plateau which is maintained for the duration of the stimulus (Millecchia and Mauro, 1969*a*).

In this study we have attempted to resolve the question of the role of IP_3 in visual excitation of microvillar photoreceptors by using pharmacological agents that block at different stages of the putative IP_3 cascade suggested by Fein (1986). In this cascade (diagrammed in Fig. 1), photoactivated rhodopsin (*RHO) activates a GTP-binding protein (G) which in turn activates PL-C. PL-C hydrolyzes PIP_2 to form IP_3 as one of its products, and IP_3 causes the release of calcium from intracellular stores; the resulting rise in Ca_i^{2+} leads to the membrane depolarization. The sites of action of neomycin, heparin, and BAPTA, the three agents used in this study to interfere with the inositol phosphate cascade, are at different steps in the cascade (Fig. 1). Therefore, similarities in their effects on the light response should reflect specific effects on the IP_3 cascade, rather than nonspecific desensitization of the cell. We show that these three agents significantly inhibit the transient component of the response to a step of light at all intensities, and that this appears to be due to a suppression of the light-induced rise in Ca_i^{2+}. We also find that the three agents fail to inhibit the plateau phase of the response to a step of light at high intensities. This latter finding is not consistent with a transduction system involving only calcium-mediated excitation. We incorporate these results into a model which suggests that the inositol phosphate cascade and a second parallel process are responsible for the production of the light response in invertebrate microvillar photoreceptors.

METHODS

Recording and Stimulation

Methods for removing and preparing the ventral nerves containing the photoreceptors of *Limulus polyphemus* have been previously described (Fein and DeVoe, 1973). Briefly, ventral nerves were dissected out, desheathed, treated with 1% pronase, and pinned to Sylgaard in the bottom of a plexiglass holding chamber containing artificial seawater (ASW; see Table I). Conventional methods of intracellular recording and voltage clamping were used, and have been described previously (Fein and Charlton, 1975, 1977). Our methods are very similar to those originally described by Millecchia and Mauro (1969*a*, *b*).

Cells were stimulated with white light from a 45-W tungsten–halogen bulb focused on the preparation (Fein and Charlton, 1975). The intensity of the unattenuated beam was 3.5 mW/cm^2, and stimulus intensities throughout this article are given as $\log_{10} I/I_o$, where I_o is the unattenuated light intensity. Cells were illuminated throughout the experiments with infrared light from a substage illuminator, allowing for visualization of the photoreceptors (and substances injected into the cells) with an infrared-sensitive video camera (Corson and Fein, 1983). Cells were impaled with two glass microelectrodes containing the substances to be injected. Injections were achieved by applying brief pressure pulses to the back of the micropipettes according to the method of Corson and Fein (1983).

Placement of Electrodes

Previous work has shown that ventral photoreceptors are segmented into two lobes: a light-insensitive arhabdomeral (A) lobe and a light-sensitive rhabdomeral (R) lobe (Calman and Chamberlain, 1982; Stern et al., 1982). Since the IP_3-sensitive calcium store and the calcium-activated conductance are located in the R-lobe (Levy and Fein, 1985; Payne et al., 1986*a*; Payne and Fein, 1987), in all experiments involving IP_3 or Ca^{2+} injections the electrodes containing IP_3 or Ca^{2+} were always placed in the R-lobes, as determined by responses to small injections (Payne et al., 1986*a*; Payne and Fein, 1987).

Chemicals and Injection Solutions

The concentration of all the substances injected into the cells are given in Table I, and represent the concentration in the injection electrode. All agents injected into the cells, with the exception of aequorin, were dissolved in a potassium–aspartate carrier solution (Table I). Control injections of this carrier had no effect. The tips of the injection pipettes were < 1 μm in diameter, and based on previous calibrations of similar electrodes (Corson and Fein, 1983), we estimate that each pressure injection delivered 5–50 pl of solution into the cell. With an average cell volume of ~500 pl (Clark et al., 1969; Stell and Ravitz, 1970; Corson and Fein, 1983), the concentrations of the chemicals in the cells were between 1 and 10% of the concentrations in the injection electrodes. Actual intracellular concentrations of the chemicals are not given because (*a*) the receptor cell is compartmentalized into two lobes; therefore, an intracellular concentration based on total cell volume would not reflect the concentration in the R-lobe, which is the region of interest, and there is no way of measuring R-lobe volume; and (*b*) there is no information about if, or how rapidly, these substances are metabolized by *Limulus* ventral photoreceptors.

TABLE I

Solutions

Solution	Concentration
ASW	435 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 25 mM $MgSO_4$, 20 mM $MgCl_2$, 10 mM HEPES
Injection solutions*	
K-asp carrier	100 mM K-asp, 10 mM HEPES
IP_3	50 μM
$Ca(asp)_2$‡	2 mM L-aspartic acid, 1 mM $Ca(OH)_2$
Heparin	1-2 mg/ml
Protamine sulfate	3 mg/ml
Neomycin	5 mg/ml
CaBAPTA	100 mM BAPTA, 50 mM $Ca(asp)_2$
BAPTA	100 mM
Aequorin	13 mg/ml, 100 mM KCl, 9 mM HEPES, 10 μM EDTA, pH 7.1

*All injected solutions except aequorin were dissolved in the K-asp carrier solution at pH 7.0.

‡By breaking our electrodes on the Sylgaard in the bottom of the holding chamber, we were able to inject the calcium solution without the addition of Triton X to prevent electrode clogging (Payne et al., 1986).

All the chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO), with the following exceptions: Neomycin sulfate was obtained from Pharma-Tek Inc. (Huntington, NY), calcium hydroxide was obtained from Mellinckrodt (Paris, KY), and protamine sulfate and BAPTA (bis-[0-aminophenoxy]-ethane-*N*,*N*,*N*^{1}, *N*^{1}-tetraacetic acid) were obtained from CalBiochem (La Jolla, CA). IP_3 was the generous gift of Dr. R. F. Irvine. Aequorin was the generous gift of Dr. O. Shimomura (NSF DIR-8801148 to O. Shimomura).

Aequorin luminescence was monitored with a photomultiplier tube with a bi-alkali photocathode (model R464; Hamamatsu, Bridgewater, NJ), operated with a photon counter (model 1770; PRA Inc., London, Ontario, Canada), which provided an analogue voltage proportional to counts accumulated every 10 ms.

Statistical Analysis

The amplitude values of the plateau and transient portions of the step response before drug introduction were subtracted from the values measured after drug introduction. Paired sample *t* tests were performed on these difference values (Zar, 1974) to determine if the effects of the drugs were significant at the 0.05 level.

RESULTS

Effects of Heparin on Responses to Brief Light Flashes

Heparin is a potent inhibitor of IP_3-induced calcium release in several systems (Hill et al., 1987; Cullen et al., 1988; Ehrlich and Watras, 1988; Ghosh et al., 1988; Kobayashi et al., 1988) and appears to function by binding tightly to IP_3-binding sites (Worley et al., 1987; Supattapone et al., 1988). If heparin blocks IP_3 receptors in *Limulus* ventral photoreceptors, then, according to the cascade of Fig. 1, injections of heparin should block the light response as well as responses to injections of IP_3, but should have no effect on responses to injections of calcium, which bypass the point of inhibition. Two sets of experiments were carried out, in which cells were simultaneously impaled with two electrodes, one of which contained heparin. The other electrode contained either IP_3 or calcium. Responses to 50-ms injections of IP_3 or calcium were repeatedly elicited to ensure the reproducibility of the responses shown. After the injection of heparin, responses to brief (30 ms) flashes of light and injections of IP_3 were depressed (Fig. 2 *A*), while responses to injections of calcium were unaffected (Fig. 2 *B*). Identical results with calcium were obtained when smaller injections of calcium were used to elicit near-threshold responses (two cells), indicating that the lack of an effect of heparin on the response to injected calcium was not due to saturation of the calcium response. Heparin also appears to reduce the amplitude of the response without appreciably altering its time course (Fig. 2 *C*). These results indicate that the effects of heparin are in accord with the model of Fig. 1, and that the concentration of heparin used is not nonspecifically desensitizing the photoreceptors.

Effects of Heparin on Calcium Release

According to the model in Fig. 1, heparin should inhibit the light-induced rise in Ca_i^{2+} by inhibiting the IP_3-induced release of calcium. The luminescent photoprotein aequorin, whose luminescence is graded with calcium concentration (Shimomura et al., 1962) has been used in several earlier studies to monitor light and IP_3-induced rises in Ca_i^{2+} in *Limulus* ventral photoreceptors (Brown and Blinks, 1974; Bolsover and Brown, 1985; Payne and Fein, 1987) and was used here to monitor the effects of heparin on the light-induced rise in Ca_i^{2+}. Cells were impaled with two electrodes, one containing aequorin and the other containing heparin. With our system, a detectable luminescent signal of reproducible amplitude from aequorin could only be produced with light intensities of $\log I/I_o = -3$ or brighter (using 30-ms flashes), which is 10 times brighter than the flash intensities used in the experiments described in Fig. 2. At this intensity the receptor potential is saturated, as can be seen by comparing Fig. 3 with Fig. 2. In Fig. 2, a $\log I/I_o = -4$ light produced a receptor potential of ~ 30

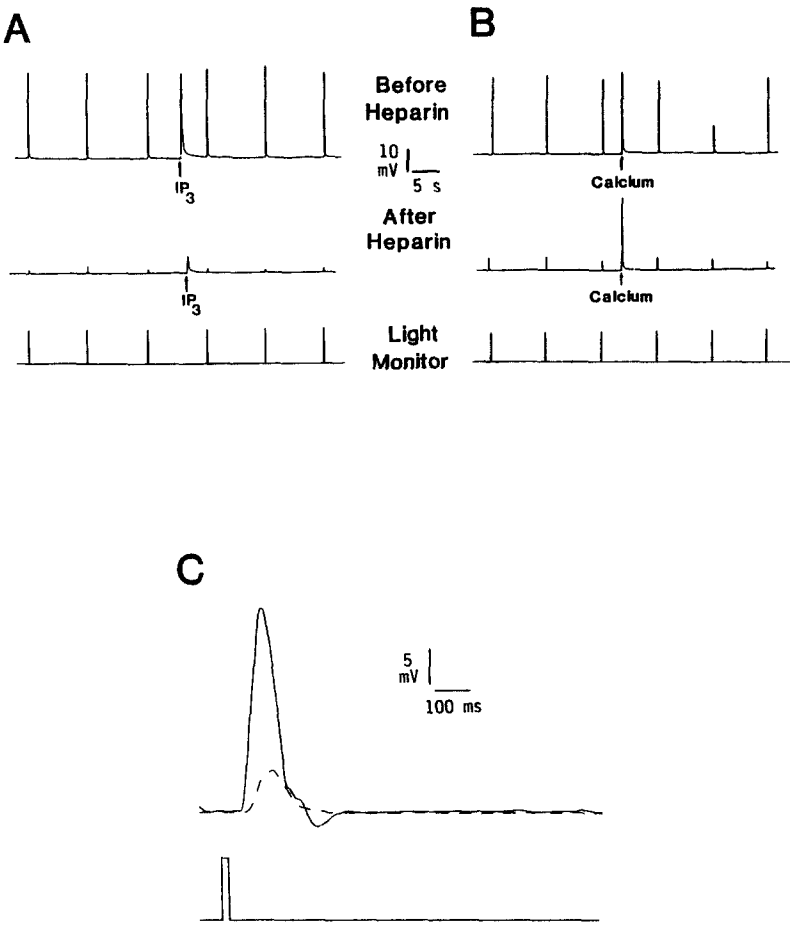

FIGURE 2. Effects of heparin on responses to flashes of light and injections of IP_3 and Ca^{2+}. (*A*) Responses to 10-ms flashes of light and an injection (5–50 pl/injection) of IP_3 before and after two injections of heparin. IP_3 was injected three times over 1-min intervals to ensure the reproducibility of the single response shown. Heparin significantly depressed the responses to both light and injected IP_3. Light intensity was log $I/I_0 = -4$. (*B*) Responses to 10-ms light stimuli and an injection of calcium before and after two injections of heparin in another cell. Heparin depressed the response to light without affecting the response to calcium. Light intensity was log $I/I_0 = -4$. (*C*) Superimposed responses to a 20-ms flash of light before (*solid line*) and after (*dashed line*) an injection of heparin in another cell, shown on an expanded time scale. The bottom trace shows the superimposed light stimuli. Heparin clearly attenuates the amplitude of the response without significantly changing its time course. Light intensity was log $I/I_0 = -4.5$.

mV, while a 10–100-fold increase in light intensity in Fig. 3 produced a receptor potential that was less than twofold greater. Because the receptor potential was saturated, larger injections of heparin were needed to see an inhibition of the light-induced depolarization. Additionally, several large injections of aequorin were

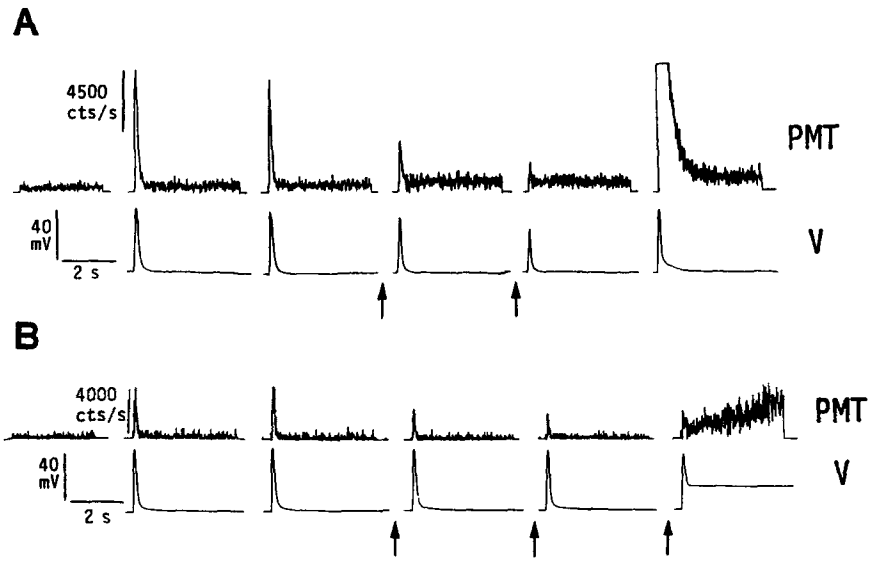

FIGURE 3. Effects of heparin on the light-induced rise in Ca_i^{2+}, monitored using aequorin luminescence. The traces labeled PMT show the photon counts detected by the photomultiplier tube after a flash of light; the traces labeled V show the membrane depolarizations produced by the same flash. Responses were elicited by 30-ms flashes of light. The shutter on the PMT opened 1 ms after the flash and remained open for 4 s to ensure that the complete time course of the aequorin luminescence was recorded. (*A*) In this cell, responses were elicited to light of log $I/I_0 = -2$. The first PMT trace is the background luminescence after the introduction of aequorin. The first arrow signifies eight heparin injections, and the second signifies five injections. The first two light-induced responses demonstrate that the amplitudes of both the aequorin luminescence and the receptor potential were stable before the introduction of heparin. After each series of heparin injections, both the receptor potential and the aequorin signal elicited by the light flash decreased, although an increase in baseline luminescence is visible. The light intensity was increased to log $I/I_0 = -1$ for the final stimulus; the large aequorin response indicates that sufficient unstimulated aequorin was available throughout the experiment to produce a response of this size if sufficient calcium was released. (*B*) In another cell, the light intensity was log $I/I_0 = -3$. The first PMT trace shows the background luminescence. Six injections of heparin occurred at the first arrow, five at the second, and four at the third. Before the introduction of heparin, the amplitudes of both the receptor potential and aequorin luminescence, elicited by a 30-ms light flash, were stable. After each series of heparin injections, both the aequorin luminescence and the receptor potential decreased (the decrease in the receptor potential was from 50 to 45 mV) without any shift in baseline luminescence. After the last injection series, a large, prolonged rise in the luminescence signal occurred, indicating that the final injection of heparin produced a rise in intracellular calcium.

required to produce a sizable luminescent signal in response to light, and these prior injections of aequorin also appeared to increase the amount of heparin required to inhibit the light-induced depolarization. This was tested by impaling other cells with the same heparin electrode and determining the number of injections required to inhibit the light-induced depolarization. In these cells, two to four 20-ms injections produced an inhibition, while in five cells previously injected with aequorin, five to

eight 20-ms injections were required. These large injections did not damage the cells, as demonstrated by their large and normal responses to light. In Fig. 3 *A*, it can be seen that responses to flashes of light were stable with respect to both the aequorin luminescence and cell membrane depolarization before the injection of heparin. After the injections of heparin, both responses decreased. While it appears that the light-induced rise in calcium is nearly abolished without an equal depression of the receptor potential, this is partly a function of the steepness of the relationship between log Ca_i^{2+} and the intensity of aequorin luminescence (Blinks et al., 1982). The steepness of this relationship means that a small decrease in the light-induced rise in Ca_i^{2+}, which leads to a small decrease in the receptor potential, will result in a large decrease in aequorin luminescence. Additionally, the rise of Ca_i^{2+} in response to light is highly localized (Payne and Fein, 1987), and such a localized rise is difficult to detect against the background luminescence of the whole cell. Also, as mentioned above, the receptor potential is saturating at these light intensities, and the small heparin-induced decrease masks a much larger decrease in the underlying light-induced current.

High concentrations of heparin appear to induce a massive release of calcium in some systems (Ritov et al., 1985), and our results in ventral photoreceptors are equivocal. In three of four cells, a rise in baseline luminescence (such as that seen in Fig. 3 *A*) was evident after heparin injection. In the other cell, the aequorin luminescence and receptor potential decreased without any change in baseline luminescence after the introduction of heparin (Fig. 3 *B*). However, after the last heparin injection, a steady rise in aequorin luminescence is evident, along with an abnormal receptor potential. These results suggest that the effect of heparin is concentration dependent, and that the initial and primary effect is to inhibit the release of calcium in response to light.

Effects of Heparin on Responses to Steps of Light

Limulus photoreceptors respond to a step of light (> 1 s) with a biphasic depolarization consisting of an initial transient followed by a steady-state plateau phase (Millecchia and Mauro, 1969*a*, *b*). We initially examined the effects of heparin on the two phases of the step response by eliciting depolarizations in response to 3-s light stimuli of intensity $\log I/I_o = -4$, and injecting heparin once the responses of the cell had stabilized. As shown in Fig. 4, heparin appears to selectively diminish the transient component without affecting the plateau component. Heparin is a fairly cytotoxic agent when used in high concentrations, so it was important to demonstrate that its effects on *Limulus* ventral photoreceptors were not simply due to cell damage. Therefore, protamine sulfate was injected into the cell through another electrode in an attempt to reverse the effects of heparin. Protamine sulfate has long been used to reverse the anticoagulant effects of heparin (Chargaff and Olson, 1939; Jorpes et al., 1939), and it has recently been found to block the inhibitory effects of heparin on IP_3-induced calcium release in vitro (Watras, J., and B. E. Ehrlich, personal communication). In *Limulus* ventral photoreceptors it also tended to reverse the effects of heparin, restoring the amplitude of the transient to the preheparin level, indicating that heparin's effects were not due to cell damage.

We next examined the effects of heparin on both phases of the step response as a

function of light intensity. A series of experiments were conducted in which the responses to 3-s steps of light were measured under voltage clamp at different intensities, before and after injections of heparin. The experiments were done under voltage clamp to avoid the complication of studying effects on both the voltage- and light-dependent conductances. The cells were impaled with two electrodes, one containing heparin and one containing 3 M KCl, allowing the cell to be voltage clamped to its resting membrane potential in the dark. Because light adaptation differentially affects the transient and plateau phases (Millecchia and Mauro, 1969*a*; Lisman and Brown, 1975*a*), a 30-ms test flash was used to monitor the level of dark adaptation in the cell. Initial responses to several 30-ms test flashes were elicited before the first response to a step of light was measured. Subsequent responses to each step flash were elicited only after the amplitude of the response to the 30-ms test flashes had recovered to the initial level. Lisman and Brown (1975*a*, *b*) found that in cells that were dark adapted, the transient portion of the voltage clamped response

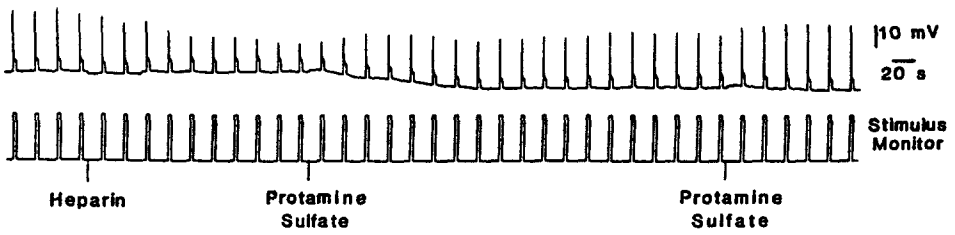

FIGURE 4. Effects of heparin and protamine sulfate on responses to 3-s steps of light. An injection of heparin depressed only the initial transient portion of the response. After the injections of protamine sulfate, the inhibitory effects of heparin are reversed and the transient recovers in amplitude. Control injections of protamine sulfate alone had no effect on the response. Note that the plateau component of the response did not change throughout the experiment. The upward deflections of the stimulus monitor indicate when the 3-s light stimuli occurred. The downward deflections designate injections of heparin or protamine sulfate as indicated. Light intensity was log $I/I_0 = -4$.

is a linear function of light intensity, except at the highest intensities, while the plateau amplitudes are a much weaker function of intensity. We obtained equivalent results with our experimental protocol (Fig. 5, *A* and *B*), demonstrating that the time allowed between the 3-s light stimuli was sufficient to maintain the cells in the same state of dark adaptation throughout the experiment. The plateau amplitudes (Fig. 5 *B*) are somewhat smaller than the amplitudes reported by Lisman and Brown for comparable intensities because we measured plateau amplitudes at the end of a 3-s step of light, while Lisman and Brown measured them at the end of a 1.1-s step of light.

Heparin depressed the amplitude of the transient at all intensities (Fig. 5 *A*), but its effects on the plateau responses were intensity dependent (Fig. 5 *B*). In the example shown, at the highest intensities (log $I/I_0 = -2$ and -3), the plateau amplitude did not change in response to the first injection of heparin, and increased slightly after the second injection (Fig. 5, *B–D*), which is another indication that heparin is not

desensitizing the cell through a rise in Ca_i^{2+}. At the "transition" intensity, the plateau remained unchanged throughout the experiment (Fig. 5, *B* and *E*), while at the lowest intensity (log $I/I_o = -5$) the plateau decreased with each injection of heparin (Fig. 5, *B* and *F*). Statistical analysis of pooled results from a total of 10 cells showed

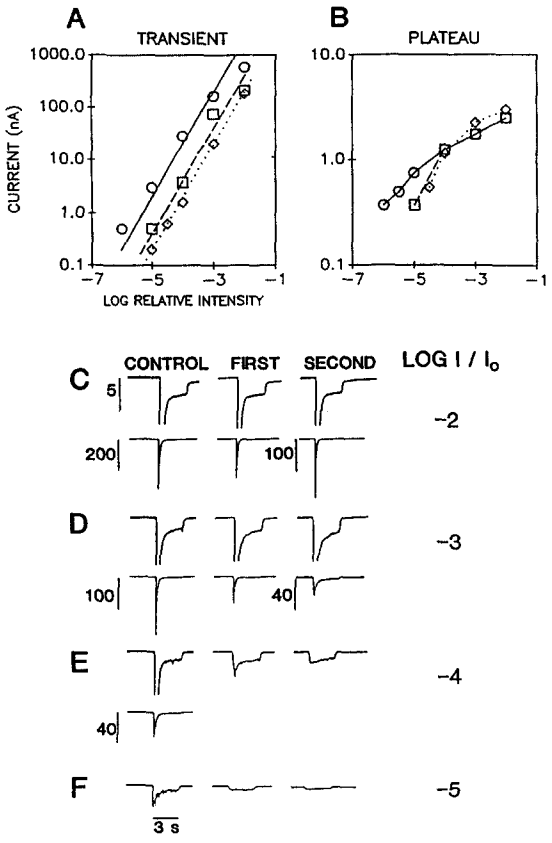

FIGURE 5. Effects of heparin on response-intensity curves and response waveforms. All data shown are from the same cell. (*A*) Peak amplitudes of the transient phase of response to 3-s steps of light of varying intensities before heparin (○), after the first injection of heparin (□), and after the second injection of heparin (◇). Heparin depressed the transient amplitudes equally at all intensities. Lines drawn through data have slopes of 1. (*B*) Amplitudes of the plateau phase of response measured at the end of the response. The first injection of heparin diminished the plateau at intensities of log $I/I_o < -4$. At intensities of log $I/I_o = -4$ and higher the plateau remained unchanged. The second injection further reduced the plateau amplitude at intensities of log $I/I_o < -4$, and slightly enhanced the plateau at intensities of log $I/I_o > -4$. (*C*) Response waveforms for 3-s steps of light at log $I/I_o = -2$. Top row shows the change in the plateau amplitudes at high amplification; the same amplification was used to display the plateau amplitudes at all the other intensities. Bottom row shows the change in the transient amplitudes using a lower amplification. Note the change in amplification for the last response. (*D*) Response waveforms for responses to 3-s steps of light of log $I/I_o = -3$. Top row shows the plateau amplitudes at high amplification; bottom row shows their respective transients at lower amplification. (*E*) Response waveforms for responses to 3-s steps of light at log $I/I_o = -4$. Top row shows the plateau portions of the response. Only one transient response is shown at the lower amplification because subsequent responses were visible in their entirety at the higher amplification. (*F*) Response waveforms for responses to 3-s steps of light at log $I/I_o = -5$. The data points in *A* and *B* were obtained from the records in *C–F*.

that the effect of heparin in suppressing the amplitude of the transient was statistically significant at all light intensities ($P < 0.05$). Additionally, heparin significantly increased the amplitude of the plateau at log $I/I_o = -2$ and -3, and significantly decreased the plateau amplitude at the lowest light intensity, log $I/I_o =$

−5. Its effects on the plateau amplitude at log $I/I_o = -4$ (the "transition" intensity) were not significant.

Due to the length of time required for cells to recover from the higher intensity steps, the preparations did not survive long enough to complete measurements for more than two injections of heparin.

Effects of Neomycin on Responses to Brief Flashes of Light

The aminoglycoside antibiotic neomycin binds to PIP_2, thereby preventing production of IP_3 (Schacht, 1976, 1978; Lodhi et al., 1980; Downes and Michell, 1981; Carney et al., 1985; Kasianowicz et al., 1988; Gabev et al., 1989), and has been shown to block calcium release thought to be mediated by IP_3 in hamster fibroblasts (Carney et al., 1985). According to the model of the inositol phosphate cascade in Fig. 1, neomycin, by preventing the degradation of PIP_2, should block the light reponse, but should not affect responses to injections of IP_3, which bypass the point of neomycin inhibition. Fig. 6 *A* shows that neomycin, as expected, blocks the light response without affecting responses to IP_3 injections. However, due to the high concentration of neomycin (5 mg/ml) used in our experiments, and to demonstrate that the lack of an effect on the response to IP_3 was not due to saturation of the response to injected IP_3, the experiment was repeated using injections of IP_3 that gave threshold responses. Fig. 6 *B* shows the responses of two cells to near-threshold injections of IP_3 (repeated several times to ensure reproducibility), before and after the introduction of neomycin. While neomycin significantly inhibited the responses to light, responses to IP_3 injections were actually slightly larger after the neomycin injection, indicating that the light flashes were probably adapting the responses to IP_3 (Brown et al., 1984*b*; Fein et al., 1984). Since light appears to adapt the response to IP_3 via a rise in Ca_i^{2+} (Payne et al., 1988), this finding suggests that neomycin inhibits the light-induced rise in Ca_i^{2+}.

Effects of Neomycin on Calcium Release

Neomycin has been found to induce calcium release in permeabilized platelets (Nakashima et al., 1987). In *Limulus* ventral photoreceptors, increases in intracellular calcium inhibit IP_3 responses (Payne et al., 1988), indicating that this is not the mode of action of neomycin in our preparation. However, because neomycin has the potential to interact with other cellular components (Prentki et al., 1986), it was important to demonstrate, as we did with heparin, that neomycin actually inhibits the light-induced rise in Ca_i^{2+} (see Fig. 1). Therefore, the effects of neomycin were also tested in the presence of aequorin. As shown in Fig. 6 *C*, neomycin produced a decrease in the light-induced aequorin luminescence along with a decrease in the receptor potential. Again, as with heparin, much larger injections of neomycin were required to produce an effect in the presence of aequorin. In contrast to heparin, however, we did not find any evidence of neomycin-induced calcium release (i.e., a sustained elevation in aequorin luminescence) in any of the five cells tested.

Effects of Neomycin on Responses to Steps of Light

We next examined the effects of neomycin on the transient and plateau phases of the step response as a function of light intensity. We attempted to generate current/

intensity graphs as with heparin, but found that the effect of small injections of neomycin on the light response did not stabilize, but continued changing over time, perhaps because the neomycin was leaking out of the pipette into the cell. Responses to steps of light could therefore not be measured over an intensity range with the cell

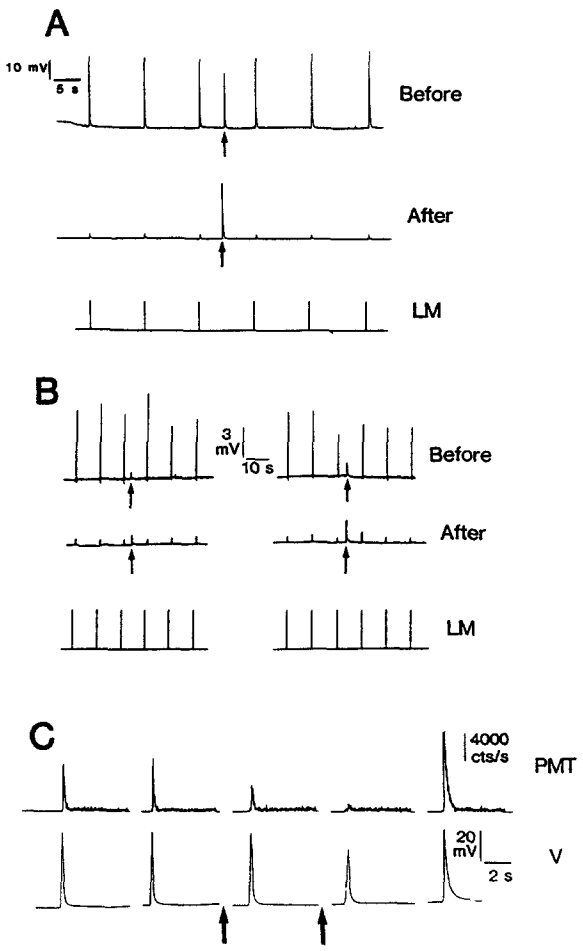

FIGURE 6. Effects of neomycin on photoreceptor responses and light-induced rise in Ca_i^{2+}. (*A*) Responses to 10-ms flashes of light and an IP_3 injection before and after the introduction of neomycin. Neomycin (two injections) diminishes the response to a brief flash of light, but does not affect the response to IP_3. Arrows designate IP_3 injections. Light intensity was log $I/I_0 = -4$. LM designates light monitor. (*B*) Responses to 10-ms flashes of light and small injections of IP_3 (eliciting threshold responses) in two cells before and after the injection of neomycin. Neomycin depresses the light responses, but IP_3 responses are slightly larger than they were before the introduction of neomycin. The arrows designate IP_3 injections. Light intensity was log $I/I_0 = -4$. (*C*) Aequorin luminescence (*PMT*) and membrane depolarization (*V*) in response to light stimuli (30 ms; log $I/I_0 = -3$) before and after the introduction of neomycin. The first PMT trace shows the background luminescence after the introduction of aequorin. Both the light-induced aequorin luminescence and membrane depolarization were stable and reproducible before neomycin injections, which are designated by the arrows. Each arrow signifies five neomycin injections. Neomycin decreased the membrane depolarization and aequorin luminescence induced by light. For the final stimulus, the light intensity was increased to log $I/I_0 = -2$; the large aequorin luminescence indicates that unstimulated aequorin was available for all the earlier responses.

in the same state of responsiveness, a critical requirement for valid response/intensity plots, and therefore an alternate strategy was adopted. Cells were impaled with two electrodes, one containing neomycin and the other KCl, and voltage clamped to their resting membrane potentials in the dark. Each cell was tested at only one intensity,

and the changes in the transient and plateau were monitored over time. To ensure that light adaptation was not a contributing factor to the results obtained, the same time intervals between step responses that successfully prevented light adaptation during the heparin experiments were used here. As shown in the examples in Fig. 7, *A–D*, neomycin diminished the amplitudes of the transient responses at all intensities, and these effects were significant ($P < 0.05$). At the higher intensities ($\log I/I_0 = -2$ and $\log I/I_0 = -3$), neomycin enhanced the amplitude of the plateau. The "transition" intensity, at which the plateau remained unchanged throughout most of the experiment, was the same as in the heparin experiments: $\log I/I_0 = -4$ (Fig. 7 *C*). At the lowest intensity ($\log I/I_0 = -5$) the amplitude of the plateau decreased throughout the experiment (Fig. 7 *D*).

At the highest intensities ($\log I/I_0 = -2$ and $\log I/I_0 = -3$) the response waveforms became distorted as the transient phase diminished (Fig. 7, *A* and *B*). Rather than exhibiting an almost square plateau, as occurs at the dimmer intensities (Fig. 7, *C* and *D*), the responses no longer had a distinct transient and/or plateau. Once responses had reached this point, no further measurements were taken.

Statistical analysis (the last measured value after drug introduction was subtracted from the control) showed that neomycin significantly depressed the transient response at all light intensities. Additionally, it significantly enhanced the amplitude of the plateau response at $\log I/I_0 = -2$ and -3, and significantly depressed the amplitude of the plateau at $\log I/I_0 = -5$. Neomycin did not have a statistically significant effect on the amplitude of the plateau response at the "transition" intensity ($\log I/I_0 = -4$).

Effects of CaBAPTA on Responses to Brief Flashes of Light

If the cascade presented in Fig. 1 is correct, a calcium chelator such as BAPTA should block the light response by preventing the rise in intracellular calcium. It has previously been shown that the chelator EGTA will reduce the amplitude of the response to a flash of light, but by also prolonging the duration of the response, the area under the response remains unchanged (Payne et al., 1986*a*, *b*). We used concentrations of calcium and BAPTA that provided a calculated free calcium concentration of 10^{-7} in the buffer solution, the same concentration that was used by previous investigators with EGTA (Chinn and Lisman, 1984; Payne et al., 1986*a*, *b*). Cells were impaled with two electrodes, one containing the buffer solution and other containing 3 M KCl, and were voltage clamped to their resting membrane potentials in the dark. As seen in Fig. 8, small injections of CaBAPTA (injections too small to be seen from a fairly sharp electrode) reduced the responses to brief light flashes in both amplitude and area under the curve at all intensities. These responses were recorded under voltage clamp to be compatible with the published EGTA experiments. In some cells, the response to a 3-s step of light was observed just after the response to a 30-ms flash was elicited (Fig. 8 *C*). The increase in the amplitude of the plateau demonstrates that CaBAPTA was getting into the cells even though the injections were too small to be seen, and indicates that the decrease in the amplitude of the responses to brief light flashes is due to CaBAPTA buffering calcium released during the light response.

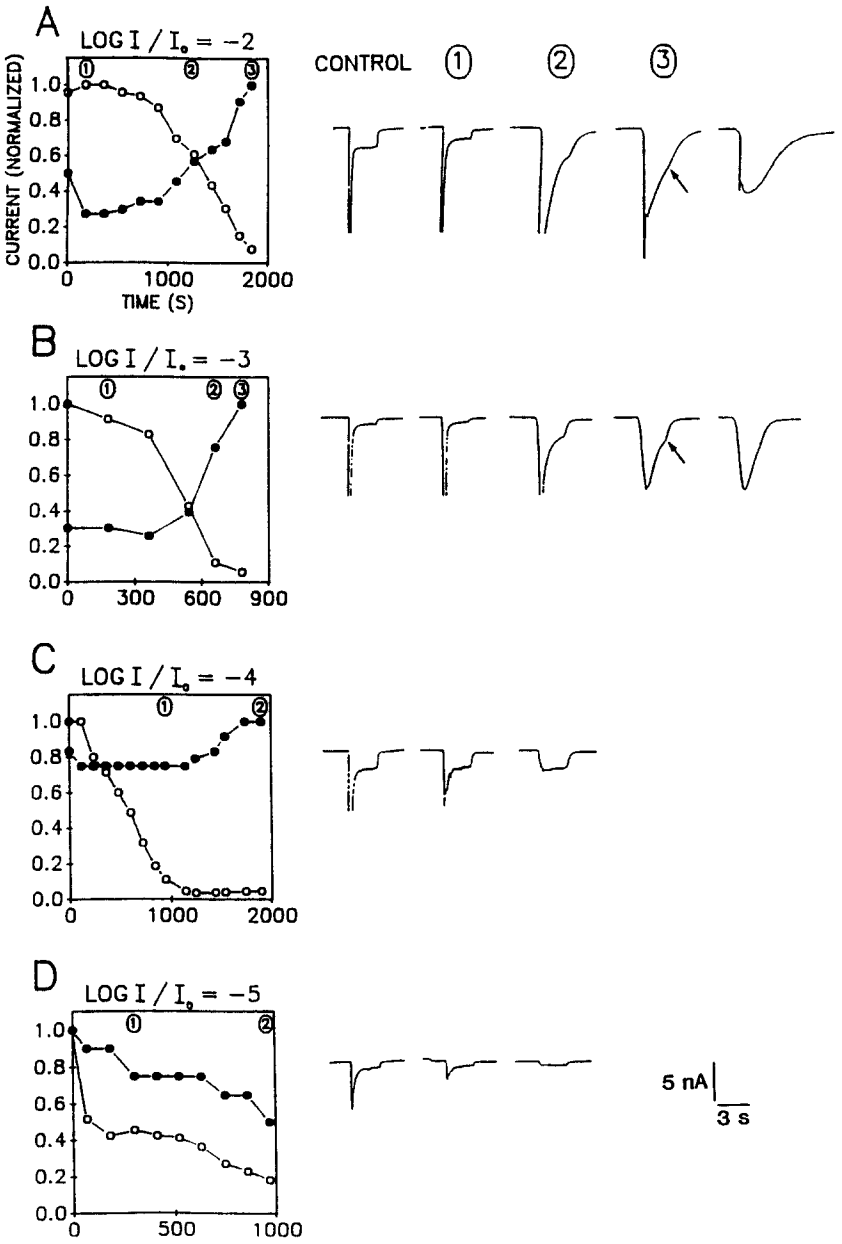

FIGURE 7. Effects of neomycin on responses to 3-s steps of light. All experiments were conducted under voltage clamp. Data are from four cells. (*A*) Transient (○) and plateau (●) amplitudes measured over time. Transient and plateau responses were normalized to their respective maxima so that they could be plotted on the same graph. At this intensity (log I/I_0 = −2) the transient decreases with time while the plateau increases. The response waveforms are from the time points designated on the graph. The control response was taken at time 0, after which neomycin was injected into the cell. The last measured plateau phase is shown by the arrow; after this, measurements were no longer taken because a distinct plateau was not visible.

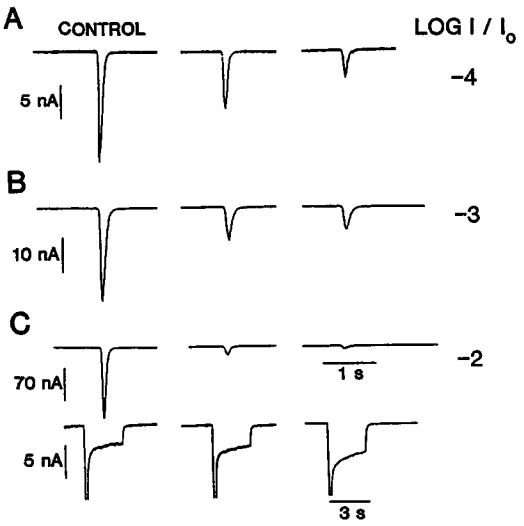

FIGURE 8. Effects of CaBAPTA on light responses. All experiments were conducted under voltage clamp. Data are from three cells. (*A*) Responses to log $I/I_0 = -4$ light flashes (30 ms) before and after several injections of CaBAPTA. The areas under the curves after CaBAPTA injections are 45 and 20%, respectively, of that of the control response. (*B*) Responses to log $I/I_0 = -3$ (30 ms) light flashes in another cell. The areas of the responses after CaBAPTA injections are 43 and 32%, respectively, of that of the control response. (*C*) Responses to log $I/I_0 = -2$ (30 ms) light flashes in another cell (*top row*). Responses to 3-s steps (*bottom row*) were recorded after the response to a 30-ms flash, with enough time between each stimulus to allow for recovery of sensitivity. Note the different time base for the step responses. Transient responses to flashes decrease, while the plateau portions of the step responses increase. Areas under the curves of responses to flashes are 19 and 8% of that of the control response.

Effects of CaBAPTA on Responses to Steps of Light

As with heparin and neomycin, voltage clamp experiments were carried out to examine the effects of CaBAPTA on the transient and plateau phases of the response to steps of light. Experiments were conducted at different intensities to determine the

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Initial and final transient amplitudes were 230 and 17.6 nA (maximal transient amplitude was 235 nA). Initial and final plateau amplitudes were 2.8 and 5.5 nA. The response waveforms in two other cells were similar to these; those of a fourth cell resembled those shown in *B*. (*B*) Normalized transient and plateau amplitudes at a lower intensity (log $I/I_0 = -3$). At this intensity, neomycin also diminishes the transient while enhancing the plateau amplitude. Axis labels are the same as in *A*. Again, the last measured plateau is shown by the arrow on the response waveform. Initial and final transient amplitudes were 175 and 9.8 nA. Initial and final plateau amplitudes were 1 and 3.3 nA. Responses in three other cell resembled these; those of a fifth cell resembled those shown in *A*. (*C*) Normalized responses at the "transition" intensity (log $I/I_0 = -4$). At this intensity the transient decreases while the plateau remains virtually unchanged throughout much of the experiment. Initial and final transient amplitudes were 70 and 3.3 nA. Initial and final plateau amplitudes were 2.5 and 3 nA. Results were the same in three other cells. In two other cells the plateau decreased near the end of the experiment. (*D*) Normalized responses at the lowest intensity (log $I/I_0 = -5$). Both the plateau and transient decreased throughout the experiment, although the effect on the plateau is not as strong as that on the transient. Initial and final transient amplitudes were 6.3 and 0.63 nA. Initial and final plateau amplitudes were 1 and 0.57 nA. Results were duplicated in four other cells.

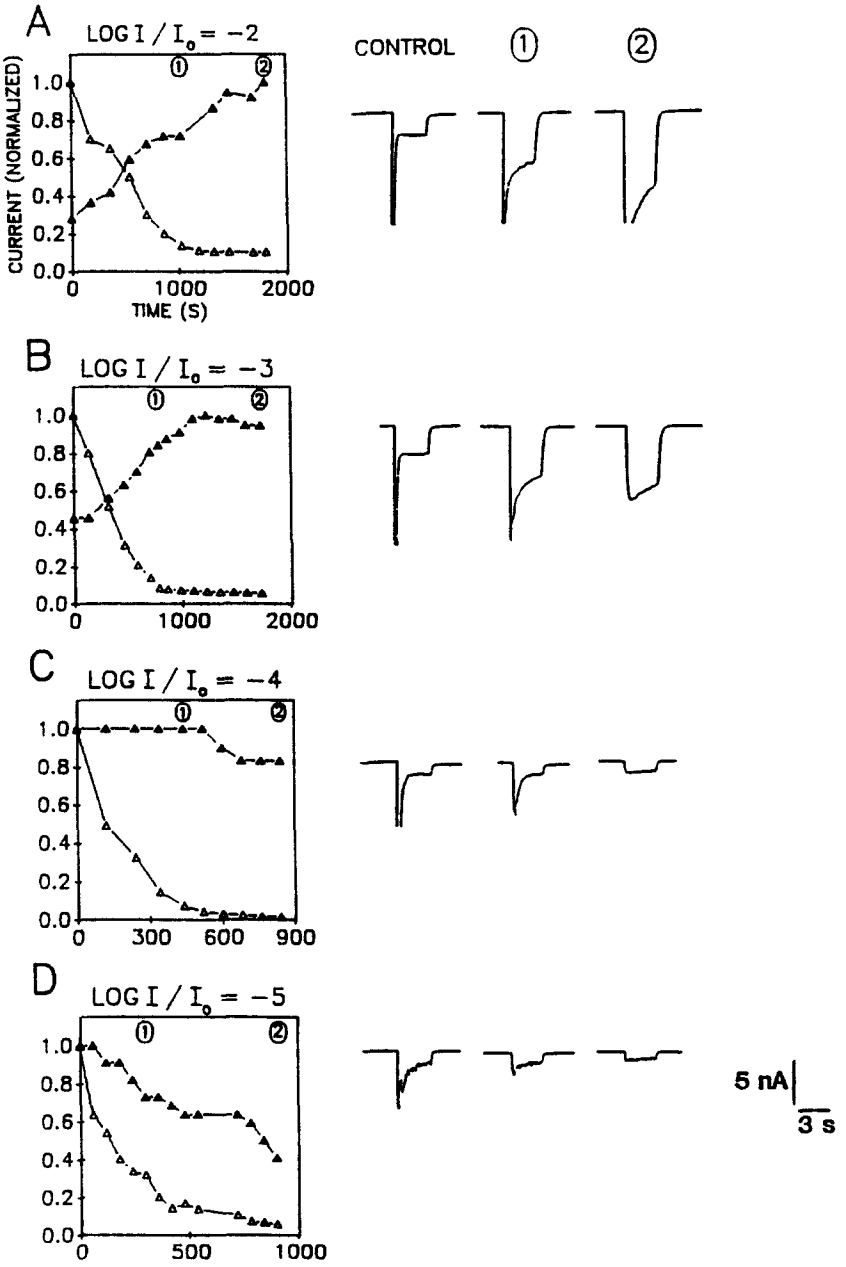

FIGURE 9. Effects of CaBAPTA on responses to 3-s steps of light. All experiments were conducted under voltage clamp. Data are from four cells. (*A*) Changes in transient (△) and plateau (▲) amplitudes monitored over time. The transient and plateau responses were normalized to their respective maxima. CaBAPTA was injected at time 0 and four other times during the experiment. At this intensity (log $I/I_0 = -2$) the transient decreases over time while the plateau increases. Response waveforms were taken at the time points designated on the graph. The control response was taken at time 0, before the introduction of CaBAPTA. Initial and final transient amplitudes were 200 and 20.8 nA; initial and final plateau amplitudes were

dependence of these effects on light intensity. Because of problems with CaBAPTA leaking out of the injection electrode (which have also been reported for the calcium chelator EGTA; Lisman and Brown, 1975*b*) these experiments were conducted in the same manner as the neomycin experiments. Cells were impaled with two electrodes, one containing CaBAPTA and the other 3 M KCl, and clamped to their resting membrane potentials in the dark, and the changes in the transient and plateau phases were monitored over time at only one intensity. At the highest light intensities ($\log I/I_0 = -2$ and -3), the plateau amplitude increased with time while the transient decreased (Fig. 9, *A* and *B*). The "transition" was again at $\log I/I_0 = -4$, where the transient amplitude decreased but the plateau amplitude remained virtually unchanged throughout the experiment (Fig. 9 *C*). At the lowest light intensity ($\log I/I_0 = -5$) both the transient and plateau responses decreased with time (Fig. 9 *D*).

Statistical analysis shows that CaBAPTA significantly diminished the amplitude of the transient response at all light intensities. At the highest intensities ($\log I/I_0 = -2$) it significantly increased the amplitude of the plateau. At $\log I/I_0 = -3$, its effects were not statistically significant. This appears to be an anomaly, since CaBAPTA produced an increase (ranging from 1 to 6 nA) in the plateau amplitudes of the four cells tested. However, with the small sample size ($n = 4$), the large variation between the cells diminished the significance of the difference. If the data are log-transformed, which is frequently done to reduce the variation, the difference is significant. At the "transition" intensity ($\log I/I_0 = -4$) CaBAPTA did not have a significant effect on the plateau amplitude, and at the lowest intensity ($\log I/I_0 = -5$) CaBAPTA significantly decreased the plateau amplitude.

While the effects of CaBAPTA on the amplitude of the plateau are very similar to those found with neomycin, the response waveforms are different at the highest intensities ($\log I/I_0 = -2$ and $\log I/I_0 = -3$). After several injections of CaBAPTA, the plateau was almost square in shape (Fig. 9 *B*), similar to the response waveforms seen after the introduction of EGTA (Brown and Blinks, 1974; Lisman and Brown, 1975*b*).

In the above experiments the BAPTA was mixed with calcium to maintain a free calcium concentration of 10^{-7} M. However, to demonstrate that the effects seen with CaBAPTA are not due to the added calcium, we conducted a similar series of

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2.8 and 5.5 nA. Similar results were obtained from two other cells. (*B*) Normalized transient and plateau amplitudes, measured at $\log I/I_0 = -3$ light intensity in another cell. CaBAPTA was injected at time 0 and three other times during the experiment. At this intensity the plateau also increases while the transient decreases. Axis labels are the same as in *A*. Initial and final transient amplitudes were 160 and 9 nA; initial and final plateau amplitudes were 2.6 and 9.25 nA. Results were reproduced in three other cells. (*C*) Normalized transient and plateau amplitudes at the "transition" ($\log I/I_0 = -4$) light intensity. CaBAPTA was injected at time 0. The plateau amplitude did not change through much of the experiment. Initial and final transient amplitudes were 90 and 1.4 nA; initial and final plateau amplitudes were 1.5 and 1.25 nA. Results were reproduced in two other cells. In two other cells, the plateau increased near the end of the experiment. (*D*) Normalized transient and plateau amplitudes at $\log I/I_0 = -5$ light intensity. CaBAPTA was injected at time 0. At this intensity both the transient and plateau decrease with time, with the transient diminishing much more rapidly than the plateau. Initial and final transient amplitudes were 6.8 and 1 nA; initial and final plateau amplitudes were 1.5 and 0.66 nA. Results were reproduced in six other cells.

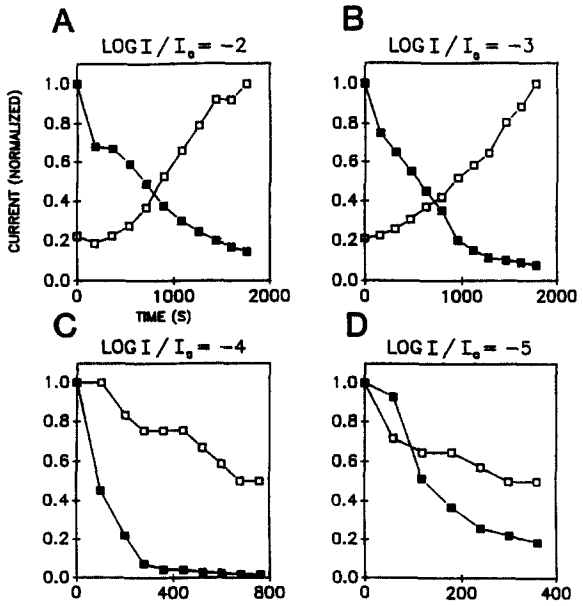

FIGURE 10. Effects of BAPTA on responses to 3-s steps of light. All experiments were conducted under voltage clamp. Due to problems with BAPTA leaking out of the electrodes, extremely sharp electrodes were used, such that injections were not visible. In all experiments injections were made between each light stimulus to ensure the continuous introduction of BAPTA. Results are from four cells. (*A*) Normalized transient and plateau amplitudes in response to steps of log $I/I_0 = -2$ light intensity. Responses were the same as those seen for CaBAPTA: the transient decreased and the plateau increased with increasing BAPTA concentration. Initial and final transient amplitudes were 300 and 43 nA; initial and final plateau amplitudes were 3.5 and 19 nA. Results were reproduced in three other cells. (*B*) Normalized transient and plateau amplitudes in response to light of log $I/I_0 = -3$. Axes are the same as those shown in *A*. Again, at this intensity, as the transient decreases, the plateau increases. Initial and final transient amplitudes were 200 and 15 nA; initial and final plateau amplitudes were 3.25 and 15.5 nA. Results were reproduced in three other cells. (*C*) Normalized transient and plateau amplitudes in response to light of log $I/I_0 = -4$. In this case, the plateau decreased slowly throughout the experiment, a result that was reproduced in four other cells, indicating that the "transition" intensity is higher under BAPTA than under CaBAPTA. Initial and final transient amplitudes were 82 and 1.3 nA; initial and final plateau amplitudes were 1.5 and 0.75 nA. (*D*) Normalized transient and plateau amplitudes in response to light of log $I/I_0 = -5$. The plateau decayed much more slowly than the transient, and this result was reproduced in four other cells. Initial and final transient amplitudes were 7 and 1.3 nA. Initial and final plateau amplitudes were 1.75 and 0.88 nA. The response waveforms are similar to those seen in the presence of CaBAPTA and are therefore not shown.

experiments using BAPTA without any added calcium. As shown in Fig. 10, the results are virtually identical to those obtained with CaBAPTA.

DISCUSSION

The Inositol Phosphate Cascade of Calcium Release

Previous work has suggested that the inositol phosphate cascade (see Fig. 1) plays a role in visual excitation of *Limulus* ventral photoreceptors (recently reviewed by Payne, 1986; Fein and Payne, 1989). The experiments presented here strongly support this suggestion since heparin (Fig. 2), neomycin (Fig. 6 *A*), and CaBAPTA (Fig. 8) suppress the transient portion of the light response, presumably by inhibiting the light-induced rise in Ca_i^{2+}. However, the results also indicate that the IP_3-

mediated pathway cannot be responsible for the whole light response, since the plateau component of the response to a step of light for light intensities above log $I/I_o = -5$ is not suppressed by these agents.

The role of IP_3-induced calcium release in invertebrate visual transduction has been the subject of much controversy. Although injections of IP_3 mimic portions of the light response (Brown et al., 1984*b*; Fein et al., 1984), the calcium buffer EGTA appeared to suppress responses to IP_3 and calcium injections without suppressing the response to light (Lisman and Brown, 1975; Rubin and Brown, 1985; Payne et al., 1986*a*), and this dilemma has long precluded the acceptance of calcium as an excitatory messenger. The data presented here with BAPTA (Figs. 8 and 9) provide the first clear evidence that calcium is necessary for generating the transient component of the light response. BAPTA has an advantage over EGTA in that it is a faster buffer (Tsien, 1980). This is particularly important when trying to suppress transient processes dependent on Ca^{2+} fluxes by using excess buffer. Neher (1985) found that EGTA was not particularly efficient in suppressing a Ca transient in chromaffin cells, while a lower concentration of BAPTA was able to suppress this current. Adler et al. (1988) also found that BAPTA produced a rapid reduction in neurotransmitter release at the squid giant synapse, while EGTA had litte effect, and they attributed this difference to the ability of BAPTA to bind calcium much more rapidly than EGTA. Therefore, BAPTA may be more efficient than EGTA in inhibiting the response to a flash of light simply because it is a faster buffer.

A New Model of Invertebrate Phototransduction

Lisman and Brown (1975*b*) found that CaEGTA has the same qualitative effects on the biphasic response to a step of light as those we report here for CaBAPTA. Based on their results, they proposed that the biphasic nature of the response was due to the decay of the initial transient to the steady-state plateau as a result of the cell light-adapting due to an increase in Ca_i^{2+}. According to their model, the action of injected CaEGTA was to prevent a rise in Ca_i^{2+}, which would inhibit light adaptation and thereby prevent the decay of the transient. The persistence of the transient amplitude would result in an apparent larger steady-state plateau phase. If their interpretation was correct, one would also expect the plateau to eventually approach the size of the initial transient, but our results with CaBAPTA show that the transient decreased to a much greater extent than the plateau increased, and that the plateau never came close to approaching the initial transient amplitude at higher light intensities. For example, at a light intensity of log $I/I_o = -2$, the initial transient was reduced from 200 to 20.8 nA with CaBAPTA, while the plateau increased from 2.8 to 5.5 nA (Fig. 9). These data suggest that the plateau is not simply the result of the decay of the transient phase, but is produced by a different mechanism.

Consequently we sought an explanation, other than that given by Lisman and Brown (1975*b*), for the transient and plateau phases of the biphasic response to a step of light. In addition, our results with heparin and neomycin indicate that the single pathway model of Fig. 1 could not account for both the transient and plateau phases. These agents are inhibitors of the pathway in Fig. 1, and we would expect them to inhibit both components of the light response if the single pathway model were correct. In reality, they actually enhanced the plateau phase at high intensities

while inhibiting the transient phase at all intensities. These considerations have led us to propose that light simultaneously initiates two separate and parallel processes that are both graded with light intensity. One of these processes is the inositol phosphate cascade (Fig. 1), while the second process operates via an unknown pathway.

Our results with heparin, neomycin, and BAPTA indicate that the inositol phosphate cascade dominates the responses to brief flashes of light, as well the transient component of the response to a step of light. All three agents inhibit responses to brief (30 ms) flashes of light, and significantly depress the transient portion of the step response at all light intensities. However, only at the lowest light intensity ($\log I/I_0 = -5$) do they significantly depress the plateau response. At light intensities greater than $\log I/I_0 = -5$, the plateau is either unchanged or significantly greater in

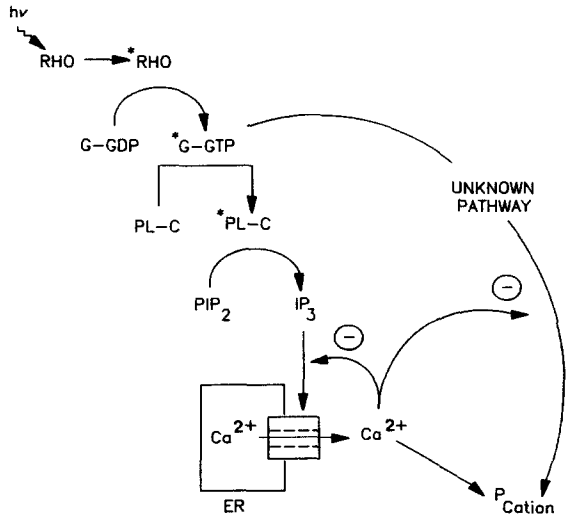

FIGURE 11. Model of visual transduction in invertebrate microvillar photoreceptors. The inositol phosphate cascade is the same as the one described in Fig. 1. IP_3 produced by this pathway releases calcium from the endoplasmic reticulum (*ER*), and this calcium either directly or indirectly, via an unknown mechanism, activates cation channels (P_{cation}) in the receptor membrane. The pathway of the second, parallel process is unknown, and may operate through the same G protein (as drawn), or have its own G protein. The two pathways are shown as separately activating membrane channels, but our data do not rule out the possibility that the two pathways come together before activating the channels and act synergistically as suggested by Payne and Fein (1986). Calcium plays a role in both excitation and adaptation via the inositol phosphate cascade, but only appears to be involved in adaptation of the second process.

the presence of these three agents. These data suggest that at low light intensities the second pathway is only weakly activated, and a portion of the plateau component results from the decay of the initial transient (as suggested by Lisman and Brown, 1975*b*), indicating that IP_3 does make a small contribution to the plateau component of the response. At higher light intensities, above the transition intensity of $\log I/I_0 = -4$, the second process dominates the plateau component, and the small contribution made by the IP_3 pathway is much less significant. These ideas are incorporated into the model of Fig. 11.

In our model, adaptation is mediated by intracellular calcium as originally proposed by Lisman and Brown (1975*b*). Calcium causes adaptation of the inositol phosphate cascade by feeding back to inhibit further calcium release by IP_3 (Payne et

al., 1988, 1990), and causes adaptation of the second process through an unknown mechanism. The enhancement of the IP_3 response in the presence of neomycin (Fig. 6*B*) is consistent with the idea that a light-induced rise in calcium inhibits the response to IP_3. According to this interpretation, calcium released during the light response inhibits further calcium release by IP_3. This inhibition would be apparent for small injections of IP_3 (Fig. 6*B*), but would not be evident for large injections (Fig. 6*A*). Reduction of calcium release by neomycin would reduce the level of adaptation, resulting in a larger response to the small IP_3 injections. Similar effects of heparin on the calcium response did not occur (Fig. 2) because increases in intracellular calcium do not appreciably inhibit responses to injected calcium (Payne et al., 1986*b*).

Adaptation of the second process by calcium released by the inositol phosphate cascade is consistent with the observation that at higher intensities, in the presence of neomycin, heparin, and BAPTA, the plateau significantly increase in amplitude as the transient amplitude decreases. At the lowest intensity, where the contribution of the IP_3 cascade to the production of the plateau is significant, the plateau amplitude decreases as the IP_3 pathway is blocked.

Lisman and Brown (1975*b*) also found that the plateau and transient responses to dim stimuli were both attenuated by the calcium buffer. They speculated that this may have been due to cell damage due to pressure injection, or because the free calcium in the buffer was providing a higher Ca_i^{2+} than would normally occur in a dimly illuminated cell, perhaps due to calcium contamination of the EGTA buffer. It is unlikely that the decrease in the plateau at the lowest light intensity is due to cell damage by pressure injection, since Lisman and Brown (1975*b*) observed the same effect upon iontophoretic injection of EGTA. The possibility that the free calcium in the EGTA or BAPTA buffers was maintaining higher internal Ca_i^{2+} than normal due to contamination by calcium from the electrode glass cannot be discounted, but Lisman and Brown (1975*b*) observed the same effect with calcium-free EGTA and we also observed the same effect with calcium-free BAPTA (Fig. 10).

Although excitation of the photoreceptor via the inositol phosphate cascade is mediated by calcium, the second pathway does not appear to require calcium for excitation, since at high light intensities the plateau amplitude actually increases in the presence of heparin, neomycin, and BAPTA.

The suggestion that another pathway is responsible for the generation of the steady-state plateau is compatible with the observations that prolonged injections of IP_3 produce only a series of transient depolarizations (Corson and Fein, 1987), rather than the biphasic response produced by a prolonged light stimulus. Payne et al. (1988, 1990) suggest that this oscillatory behavior is due to a negative feedback cycle of IP_3-induced calcium release, depolarization, and inhibition of IP_3-induced calcium release due to elevated Ca_i^{2+}, with a new cycle beginning once the period of inhibition (3–10 s) is over. These studies support our suggestion that IP_3 is responsible for the transient portion of the light response.

An earlier model by Payne and Fein (1986) proposed that light activates two parallel cascades of reactions, in which particles released by the first open ionic channels, and calcium produced during the second accelerates the rate of production of particles by the first. This model was based on available evidence at the time, which

indicated that calcium could not excite the cell (Brown and Lisman, 1975), and that calcium released by light is neither sufficient nor necessary for the generation of the photocurrent by light. A later study by Payne et al. (1986*a*) demonstrated that pressure injection of calcium is sufficient to excite the cell, and our evidence with BAPTA suggests that it is necessary for the transient portion of the response. However, the idea that calcium released during the inositol cascade may accelerate the reactions of the second pathway cannot be discounted, and warrants further study.

While the pathway of the second process is unknown, there is some indication that it involves a G protein, as does the inositol phosphate cascade (Fein, 1986), since the entire response to a step of light appears to be inhibited by GDPβS, a known inhibitor of G proteins (Kirkwood et al., 1989, Fig. 1 *D*). Both processes may operate through the same G protein, but studies in squid photoreceptors indicate that rhodopsin activates two different G proteins (Tsuda, 1987; Robinson et al., 1988), raising the possibility that each process may have its own unique G protein.

If a second process is present, an obvious candidate for the transmitter is diacylglycerol (DAG), which is produced together with IP_3 from the cleavage of PIP_2, and is a known activator of protein kinase C (Ku et al., 1981; Nishizuka, 1984*a*, *b*). However, by blocking the breakdown of PIP_2, neomycin inhibits the production of both IP_3 and DAG, but the amplitude of the plateau component at high intensities actually increases in the presence of neomycin. This indicates that DAG, resulting from PIP_2 hydrolysis, is not required for the production of the plateau. We also tested the effects of a synthetic DAG analogue (1-oleoyl-2-acetyl-glycerol), as well as several protein kinase inhibitors (H-7, staurosporine, sphingosine), but found no selective effects on the plateau component of the biphasic response (unpublished observations). However, the response waveforms in the presence of neomycin are different from those produced in the presence of heparin and BAPTA. Heparin (Fig. 5) and CaBAPTA (Fig. 9) both left a distinct steady-state plateau, while treatment with neomycin ultimately produced a response that resembled neither the transient nor the plateau (Fig. 7). These results indicate that while DAG is not the transmitter in the second cascade, it may be required for the production of a normal plateau.

Currently, the only available candidate for the transmitter of the second pathway is cGMP, which produces a membrane current with a reversal potential similar to that of the light-induced current when injected into *Limulus* ventral photoreceptor cells (Johnson et al., 1986). Responses to injections of cGMP are also not blocked by EGTA (Johnson et al., 1986), and levels of cGMP reportedly increase in squid photoreceptor preparations in the presence of light (Saibil, 1984; Johnson et al., 1986), although similar increases have not been seen in *Limulus* ventral photoreceptors (Brown et al., 1984*a*). Although the potency of cGMP is significantly lower than that of IP_3 (Feng et al., 1991), it is the only substance other than IP_3 and calcium that has been found to produce a membrane current with the same reversal potential as that of the light-induced current, and certainly warrants further study.

Phototransduction mutants in the fly may prove to be valuable for identifying the components involved in the putative second process. The *Drosophila* transient receptor potential (trp) mutant and the *Lucilia* no steady state (nss) mutant both show normal responses to dim steps of light, but at higher intensities the transient decays

quickly to baseline with no visible steady state (Cosens and Manning, 1969; Minke, 1982; Howard, 1984). The time it takes to recover from an intense light stimulus is also prolonged in both these mutants (Minke, 1982; Barash et al., 1988); therefore, Minke (1982) has suggested that these unusual responses may be due to depletion of some substance required for phototransduction. We favor an alternate explanation in which these mutants are missing some critical component of the putative second pathway. However, differences between the effects of lanthanum on *Limulus* ventral photoreceptors and *Calliphora* photoreceptors suggest that the two receptor systems may not be comparable. In *Calliphora,* application of lanthanum produces electrophysiological responses that are similar to those of the *Drosophila* trp-mutant (Hochstrate, 1989), while in *Limulus,* lanthanum has no selective effects on the plateau component of the response (unpublished observations).

Recent molecular characterization of the *Drosophila* trp gene shows that it encodes a protein that is not present in the trp mutants, indicating that the phenotype arises from the absence of this protein (Montell and Rubin, 1989; Wong et al., 1989). This protein appears to be a new component in phototransduction, as its sequence did not resemble that of any protein of known function (Montell and Rubin, 1989; Wong et al., 1989). It will be interesting to see if this protein is a critical component in the putative second pathway leading to the production of the plateau response.

In conclusion, the results presented in this paper provide substantial support for the role of IP_3-induced calcium release in invertebrate visual excitation, and appear to resolve some of the most damaging evidence against it. Our results indicate that the IP_3 cascade plays a significant role in the production of transient responses. However, it is also clear that the story is far from complete, as these results suggest that a second pathway, not mediated by IP_3, is also present and plays a significant role in the production of the steady-state response.

We thank Dr. R. Sha'afi for suggesting the use of neomycin; Drs. B. Ehrlich, L. Jaffe, J. Lisman, R. Payne, and J. Watras for providing helpful comments on an earlier version of this manuscript; and Dr. J. Chapman for help with statistics.

This work was supported by National Institutes of Health grant EY-03793.

Original version received 4 November 1989 and accepted version received 14 November 1990.

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