Intracellular Injection of Heparin and Polyamines

Effects on Phototransduction in Limulus Ventral Photoreceptors

MITCHELL N. FADDIS and JOEL E. BROWN

From the Department of Ophthalmology and Visual Sciences, Washington University School of Medicine, St. Louis, Missouri 63110; and Department of Ophthalmology and Visual Science, Albert Einstein College of Medicine, Bronx, New York 10461

ABSTRACT Heparin is thought to inhibit InsP$_3$ binding to receptors involved in the intracellular release of Ca^{2+}. Injection of heparin into *Limulus* ventral photoreceptors to high intracellular concentrations reduces the amplitude and slows the rate of rise of voltage-clamp currents induced by brief flashes, tends to make the responses to long flashes more "square," and tends to block the light-induced rise in [Ca^{2+}]$_i$ detected by arsenazo III. In these ways, intracellular heparin mimics the effects of high concentrations of intracellular BAPTA or EGTA. In addition, the effects of heparin are attenuated by prior injection of BAPTA to high intracellular concentrations. Neomycin and spermine are thought to inhibit phospholipase C activity. Injections of spermine or neomycin to low intracellular concentrations largely mimic the effects of intracellular heparin. These findings suggest that the predominant effect of polyamines is to inhibit light-induced production of InsP$_3$ by phospholipase C activity and thereby reduce the light-induced increase in [Ca^{2+}]$_i$. Our findings suggest that excitation can proceed in the absence of InsP$_3$-induced increases in [Ca^{2+}]$_i$, but (*a*) the gain and speed of transduction are reduced and (*b*) adaptation is largely blocked.

INTRODUCTION

Most invertebrate photoreceptors respond to light stimulation with a graded depolarization (called the receptor potential). In *Limulus* ventral photoreceptors, the receptor potential is produced by the opening of a cationic conductance in the photoreceptor plasma membrane (Millecchia and Mauro, 1969a; Brown and Mote, 1974). The opening of this conductance is mediated by a cascade of intracellular reactions, the phototransduction cascade, which is initiated by photoactivation of rhodopsin molecules. The molecular components of the phototransduction cascade

Address correspondence to Dr. Joel E. Brown, 506 Kennedy Center, Albert Einstein College of Medicine, 1410 Pelham Parkway, Bronx, NY 10461.

in invertebrates are not completely known (reviewed in Bacigalupo, Johnson, Robinson, and Lisman, 1990). However, the observation that rhodopsin activation stimulates phosphatidylinositolbisphosphate (PtdInsP$_2$) hydrolysis in photoreceptors in Limulus ventral eye (Brown, Rubin, Ghalayini, Tarver, Irvine, Berridge, and Anderson, 1984; Faddis, 1992), squid (Szuts, Wood, and Fein, 1986; Brown, Watkins, and Malbon, 1987), and fly (Devary, Heichal, Blumenfeld, Cassel, Suss, Barash, Rubinstein, Minke, and Selinger, 1987) suggests that PtdInsP$_2$ hydrolysis may be an important component of the cascade in most invertebrate photoreceptors.

In Limulus ventral photoreceptors, several lines of evidence suggest that the product of PtdInsP$_2$ hydrolysis, myo-inositol 1,4,5-trisphosphate (Ins(1,4,5)P$_3$), can modulate or mediate excitation of photoreceptors through a mechanism linked to a rise in intracellular calcium. (a) Intracellular pressure injection of Ins(1,4,5)P$_3$ (Brown et al., 1984; Fein, Payne, Corson, Berridge, and Irvine, 1984) or calcium (Payne, Corson, and Fein, 1986) into the rhabdomeral lobe of ventral photoreceptors opens an ionic conductance with a reversal potential similar to that of the light-induced conductance. (b) Injection of Ins(1,4,5)P$_3$ into the rhabdomeral lobe releases calcium from intracellular stores (Brown and Rubin, 1984; Payne and Fein, 1987). (c) Intracellular injection of calcium buffers largely blocks excitation produced by a subsequent injection of Ins(1,4,5)P$_3$ (Rubin and Brown, 1985; Payne, Corson, Fein, and Berridge, 1986). Also, flash photolysis of myo-inositol 1,4,5-trisphosphate-P$^{4(5)}$-1-(2-nitrophenyl)ethyl ester (caged Ins(1,4,5)P$_3$) enhances the light-induced current of desensitized ventral photoreceptors; this effect is attenuated by prior injection of the calcium chelator BAPTA ([1,2-bis-[o-amino-phenoxy]ethane-N-N-N'-N'] tetraacetic acid) (Faddis and Brown, 1992a, b).

The role of Ins(1,4,5)P$_3$-induced calcium release in modulating or mediating either light-evoked excitation or adaptation of Limulus ventral photoreceptors is unclear. In response to a prolonged stimulus, the light-induced current in Limulus ventral photoreceptors has an initial transient that decays (over several tens of milliseconds) to a plateau. Intracellular injection of a calcium buffer produces two effects that tend to make the waveform of the light-induced current more square, presumably as a result of stabilizing intracellular ionized calcium ([Ca^{2+}]$_i$). (a) The initial transient in response to a step of light is diminished in amplitude and (b) the amplitude of the plateau (induced by bright stimuli) tends to be increased (Lisman and Brown, 1975; Frank and Fein, 1991). The latter of these two effects is evidence that light-evoked desensitization (i.e., light adaptation) is attenuated by the intracellular calcium buffer (Lisman and Brown, 1975); an increase in [Ca^{2+}]$_i$ is probably an obligatory step in the pathway of light adaptation in many invertebrate photoreceptors (reviewed in Brown, 1986). On the other hand, the rising phase of the light-induced current in response to bright light flashes is accelerated by an increase in [Ca^{2+}]$_i$ (Brown and Lisman, 1975; Payne and Fein, 1986). Moreover, the light-induced current is amplified by an increase in [Ca^{2+}]$_i$ (Bolsover and Brown, 1985) in photoreceptors in which an intracellular calcium pool has been depleted. These observations suggest that [Ca^{2+}]$_i$ may participate in a nonlinear mechanism that accelerates and amplifies the output of the transduction cascade. This nonlinear mechanism may be modeled as either positive feedback or positive "feed-forward" (as suggested by Payne and Fein, 1986). Frank and Fein (1991) reported that intracellular injection of heparin,

an inhibitor of Ins(1,4,5)P$_3$ binding (Worley, Baraban, Supattapone, Wilson, and Snyder, 1987), reduced the amplitude of the initial transient response to light and attenuated the light-induced increase in [Ca^{2+}]$_i$ similar to the effects observed with BAPTA; however, they reported little effect of intracellular heparin either on the kinetics of the flash response or on the plateau amplitude of the response to a prolonged stimulus, unlike the effects of intracellular BAPTA. Frank and Fein (1991) also reported that intracellular injections of neomycin, a polyamine antibiotic, attenuated the responses to brief flashes, attenuated the light-induced increase in [Ca^{2+}]$_i$, and slowly altered the amplitude of the plateau phase of the responses to prolonged illumination.

In this study, we report that intracellular injections of solutions of heparin and the polyamines spermine and neomycin attenuate the contribution of Ins(1,4,5)P$_3$-induced calcium release to the mechanisms of excitation and adaptation in ventral photoreceptors. Our results suggest (a) that phototransduction can proceed in the absence of Ins(1,4,5)P$_3$-induced calcium release inside the cell, (b) that Ins(1,4,5)P$_3$-induced calcium release increases the gain of the phototransduction cascade and speeds the kinetics of the light response, and (c) that Ins(1,4,5)P$_3$-induced calcium release may be the principal mechanism by which light increases [Ca^{2+}]$_i$ to mediate light adaptation.

METHODS

Electrical Recording and Stimulation

The methods for dissection and voltage clamp of single photoreceptors from the ventral rudimentary eye of *Limulus polyphemus* were similar to those used by Millecchia and Mauro (1969a,b). Single ventral nerves were desheathed, pinned with glass fibers to Sylgard 184 (Dow Corning Corp., Midland, MI) in a perfusion chamber, and briefly (60 s) bathed in 2% Pronase E (Protease XXV, P6911; Sigma Chemical Co., St. Louis, MO) in artificial sea water (ASW) followed by extensive rinsing with ASW. ASW contained (mM) 423 NaCl, 10 KCl, 23 MgCl$_2$, 25 MgSO$_4$, 10 CaCl$_2$, and 10 Tris-Cl adjusted to pH 7.8. In some experiments, CaCl$_2$ was replaced mole for mole with MgCl$_2$ and 5 mM ethylene glycol-bis-(β aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) was added (low Ca^{2+} ASW); we estimate (Schoenmakers, Visser, Flik, and Theuvenet, 1992) that the free Ca^{2+} was $< 10^{-8}$ M in low Ca^{2+} ASW. During experiments, the preparation was continuously superfused with ASW at room temperature (22–23°C) at a rate of 2 ml/min. Cells were voltage clamped with two microelectrodes (Millecchia and Mauro, 1969b; Brown, Harary, and Waggoner, 1979). Microelectrodes typically had a resistance of 5–8 MΩ when filled with 2 M KCl. Experiments were performed on the stage of an inverted microscope. The preparation was transilluminated with infrared light ($\lambda = 950$ nm) from a light-emitting diode and viewed with a CCD video camera. The video signal from the camera was enhanced (IV-530 Contour Synthesizer; FOR-A Company Ltd., Tokyo, Japan) and displayed on a video monitor.

Photoreceptors were stimulated by the collimated output of a tungsten-halogen light source. The stimulus beam passed through a water cell heat filter, neutral density filters, and an electromechanical shutter into the epifluorescence light port of the microscope. The stimulus beam was focused onto the preparation from below through the microscope objective. The stimulus beam produced one quantum bump per second on average (presumably equal to one photoexcited rhodopsin per second; Fuortes and Yeandle, 1964) when attenuated 7.8 log units

with neutral density filters. The unattenuated stimulus beam energy was 1.4 mW/cm^2 measured in the plane of the preparation.

Intracellular Injection

Intracellular injections were made by applying brief (50 ms) pressure pulses (20–50 lb/in^2) to the back of microelectrodes filled with injection solution. A successful intracellular injection produced an optical disturbance in the cytoplasm of the cell clearly visible on the video monitor. Injection pressure was adjusted to produce the smallest injection that could be reliably seen. Injections meeting this visual criterion have been measured to be 1.3 ± 0.3 pl (mean ± SD, n = 5) (Faddis and Brown, 1992b). These measurements were made by including $^{35}SO_4$ at a known specific activity in the injection solution; after injection the preparation was dissolved and counted in a liquid scintillation counter and the injected volume was calculated (Brown and Blinks, 1974; Coles and Brown, 1976). The average volume of a ventral photoreceptor cell has been estimated to be ~4–5 × 10^{-10} liters based on its geometry (Clark, Millecchia, and Mauro, 1969; Corson and Fein, 1983). We did not measure the injected volumes nor did we determine the geometry for each cell reported in this paper. Therefore, a single intracellular "injection" refers to a volume of injection solution estimated to be ~0.2–0.3% of the volume of a typical ventral photoreceptor.

All compounds (except BAPTA) were dissolved in injection buffer made of 300 mM potassium acetate and 10 mM MOPS (morpholinopropane sulfonic acid), pH 7.3. BAPTA injection solution contained 300 mM BAPTA (tetrapotassium salt) and 10 mM MOPS, pH 7.3. Compounds injected were: neomycin sulfate, N,N'-bis[3-aminopropyl]-1,4-diaminobutane tetrahydrochloride (spermine), N-[3-aminopropyl]-1,4-diaminobutane trihydrochloride (spermidine), 1,4-diaminobutane dihydrochloride (putrescine), arginine-lysine dipeptide (Arg-Lys), heparin (grade II, from porcine intestinal mucosa, #H7005), de-N-sulfated heparin (#D4776), chondroitin sulfate B, and myo-inositol 1,4,5-triphosphate from Sigma Chemical Co., and BAPTA from Molecular Probes, Inc. (Eugene, OR).

Measurement of Intracellular Calcium Transients

Intracellular calcium transients were monitored with the calcium indicator dye, arsenazo III (Brown, Brown, and Pinto, 1977). 100 mM arsenazo III (Sigma Chemical Co.) in injection buffer was passed through a "calcium sponge" column (Meyer, Holowka, and Stryer, 1988) that had been previously equilibrated with injection buffer. The column eluent was diluted 1:4 (vol/vol) with injection buffer and filtered. This solution was used for dye injections; it was stored at 4°C for up to several months.

The absorption of arsenazo III injected into single photoreceptors was monitored by the transmission of a 670-nm light from a laser diode source (model LAS-200-670; LaserMax, Inc., Rochester, NY) driven by a regulated power supply (model CK8-5; Kepco Inc., Flushing, NY). This wavelength is close to the maximum (660 nm) of the difference spectrum for arsenazo III bound to calcium ions measured in ventral photoreceptors (Brown et al., 1977). The surface of the chamber was partially covered by a glass coverslip so that the measuring beam did not pass through an air–water interface. An aperture positioned in the image plane of the microscope objective restricted the light reaching a photodiode to that passing through a photoreceptor loaded with arsenazo III (Brown et al., 1977). Approximately the entire image of the cell was focused onto the photodiode. Light transmission measured by the photodiode (PV 100; EG & G Electro-Optics, Salem, MA) was filtered at 100 Hz and the change in absorbance was calculated as described previously (Harary and Brown, 1984).

RESULTS

Heparin Injection Reduces the Amplitude and Alters the Kinetics of the Light-induced Current

Unfractionated heparin, a mixture of anionic polysaccharide chains of variable length, has been found to be a competitive inhibitor of $Ins(1,4,5)P_3$-induced calcium release (e.g., Cullen, Comerford, and Dawson, 1988; Nilsson, Zwiller, Boynton, and Berggren, 1988). Moreover, heparin has no effect on the degradation of $Ins(1,4,5)P_3$ by 5-phosphatase activity in rat brain (Worley et al., 1987). De-N-sulfated heparin (Tones, Bootman, Higgins, Lane, Pay, and Lindahl, 1989) and chondroitin sulfate (Worley et al., 1987) do not inhibit $Ins(1,4,5)P_3$ binding to membrane receptors. These findings suggest that heparin may be a relatively selective antagonist of $Ins(1,4,5)P_3$ binding to calcium release receptors. Therefore, we injected heparin (as

TABLE I

Effect of Heparin and Related Compounds on the Amplitude of the Light-induced Current

Substance injected	Concentration in pipette	% Control amplitude Mean ± SD (n)
	mg/ml	
Heparin	1	101.1 ± 12.0 (3)
Heparin	10	79.1 ± 15.5 (4)
Heparin	100	20.4 ± 17.9 (7)
Heparin + BAPTA	100	76.3 ± 14.0 (6)
De-N-sulfated heparin	100	91.2 ± 10.0 (3)
Chondroitin sulfate B	100	84.7 ± 6.9 (3)

For each cell, the response amplitude after injection was normalized by dividing by the response amplitude before injection (one to three heparin injections or two to eight injections of chondroitin sulfate B or de-N-sulfated heparin). The mean normalized amplitude after injection is tabulated as % control amplitude ± SD; number of cells tested (n).

well as de-N-sulfated heparin and chondroitin sulfate as controls) intracellularly in order to examine the participation of $Ins(1,4,5)P_3$-induced calcium release in generating the light-induced current.

Ventral photoreceptors were dark-adapted a minimum of 30 min after dissection, voltage-clamped to their resting potential in the dark, and repetitively stimulated (20 ms duration flashes, one every 30 s) with a light intensity in the linear region of the stimulus vs. response relation. Only cells that responded stably to the train of repetitive stimuli were used for the following analysis. After response amplitudes had stabilized, each photoreceptor was injected with a solution of heparin or other compound (one to three injections for heparin solutions or two to eight injections for de-N-sulfated heparin or chondroitin sulfate B solutions). The data from experiments of this kind are summarized in Table I. Only responses from cells injected with 100 mg/ml heparin in injection buffer were very different from responses recorded before injection or from cells injected with either 100 mg/ml de-N-sulfated heparin

or 100 mg/ml chondroitin sulfate B. This finding is different from those reported in a previous study in *Limulus* photoreceptors in which significant reductions in the amplitude of the flash response were seen after injections of a 1 mg/ml heparin solution (Frank and Fein, 1991).

In addition to reduction in the amplitude of the light-induced current, a prominent effect of injected heparin is alteration of the rising phase of the light-induced current (whereas the falling phase remains relatively unchanged). Fig. 1 *A* illustrates typical light-induced currents recorded from a photoreceptor into which a solution of
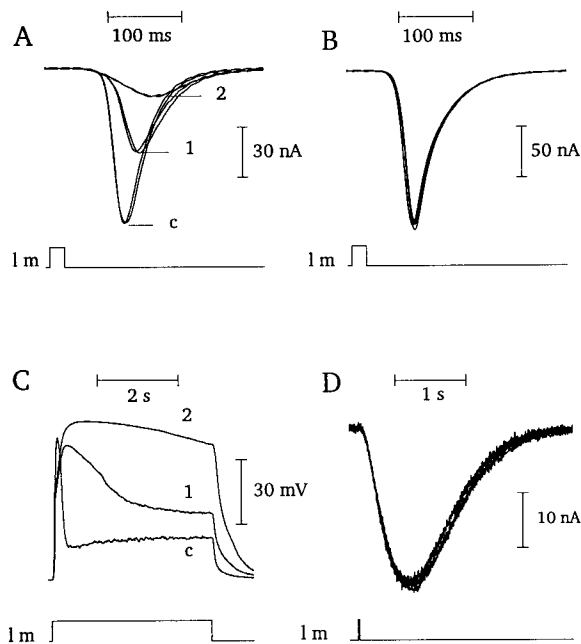

FIGURE 1. Effects of heparin injection on the waveform and amplitude of the light response. (*A*) Six consecutive light-induced currents in response to 20-ms flashes (one every 30 s, 2.4 log attenuation): two before injection, two after one injection of 100 mg/ml heparin, and two after a second injection of heparin solution. (*B*) Six consecutive light-induced currents in response to 20-ms flashes (one every 30 s, 2.4 log attenuation) before and after injections of a 100 mg/ml de-*N*-sulfated heparin solution. (*C*) Example of BAPTA loading protocol. Three receptor potentials in response to 4-s flashes (one every 60 s, 2.4 log attenuation), one before BAPTA loading, one during BAPTA loading, and one after BAPTA loading. This cell was impaled with a single microelectrode filled with 300 mM BAPTA, and 10 mM MOPS, pH 7.3. BAPTA solution was injected until the receptor potential waveform became approximately square. (*D*) Six consecutive light-induced currents in response to 20-ms flashes (one every 30 s, 1.2 log attenuation) from a BAPTA-loaded cell before and after each of five injections of a 100 mg/ml heparin solution (one per interflash interval). Horizontal scale bar: 100 ms for *A* and *B*; 2 s for *C*; 1 s for *D*. Vertical scale bars are 30 nA (*A*), 50 nA (*B*), 30 mV (*C*), and 10 nA (*D*). *A–D* were each taken from a different cell.

100 mg/ml heparin was injected. The figure shows six consecutive records of light-induced currents: two before injection, two after the first injection, and two after the second injection. After a single injection of heparin solution, the light-induced current was reduced in amplitude; in addition, the later portions of the falling phases of the responses approximately coincide with those of control responses, whereas the rising phases of the waveforms do not coincide. These observations suggest that heparin is not simply a competitive inhibitor of the ligand that gates the light-

activated channel; a competitive inhibitor would be expected to inhibit all phases of the light-induced current to a similar extent.

Unlike heparin, de-N-sulfated heparin was ineffective when injected in a 100 mg/ml solution. Fig. 1 B shows six consecutive records of light-induced current from a particularly stable cell that received a total of six injections of de-N-sulfated heparin solution: one after the first record, three after the third record, and two after the fifth record. There was no change in the amplitude or kinetics of the responses after the injections. Similar results were obtained from cells injected with a 100 mg/ml solution of chondroitin sulfate B.

To examine further if heparin was acting by attenuating an InsP$_3$-induced increase in $[Ca^{2+}]_i$, we attempted to reduce any increases in $[Ca^{2+}]_i$ by intracellular injection of the calcium sequestering agent, BAPTA. Injections of heparin were made into cells that had been previously injected with BAPTA. Cells were stimulated with long flashes of sufficient intensity to evoke responses with a prominent transient-to-plateau waveform. Between flashes, a number of injections of BAPTA solution were made. Injections were discontinued when the waveform of the responses became approximately square (Fig. 1 C). After BAPTA injection, cells were impaled with a second microelectrode that contained heparin solution, voltage-clamped to their dark membrane potential, and stimulated until the amplitude of the light-induced currents became stable. Previous studies have demonstrated that in the presence of a high intracellular concentration of calcium buffer the kinetics of the light-induced current are greatly slowed (Lisman and Brown, 1975; Payne and Fein, 1986) and the sensitivity is reduced (Lisman and Brown, 1975; Frank and Fein, 1991); data obtained in this study are consistent with those observations. After the light-induced current amplitude became stable, the cell was injected with heparin solution. Fig. 1 D shows a set of six consecutive light-induced currents from a particularly stable BAPTA-loaded cell during heparin injection. Unlike the result in Fig. 1 A, there was no change in the light-induced current after each heparin injection. The result illustrated in Fig. 1 D and Table I (for $n = 6$ cells) is consistent with the idea that intracellular heparin tends to attenuate the light-evoked rise in intracellular calcium; in BAPTA-loaded cells, the light-evoked rise in intracellular calcium had already been attenuated before heparin injection.

Heparin Injection Attenuates the Light-induced rise in $[Ca^{2+}]_i$

In *Limulus* photoreceptors, the decline of the transient phase to the plateau during constant photon flux is evidence for the process of light adaptation. Intracellular injection of a calcium buffer to a concentration that substantially attenuates the light-evoked rise in $[Ca^{2+}]_i$ produces a receptor potential that is approximately square (Brown and Blinks, 1974; Brown et al., 1977). This observation is strong evidence that the light-evoked rise in $[Ca^{2+}]_i$ mediates the process of light adaptation (for review see Brown, 1986). Frank and Fein (1991) reported that injections of a solution of heparin reduced the light-evoked release of calcium, suggesting that Ins(1,4,5)P$_3$-evoked calcium release contributes to the total calcium released by light. However, the extent to which Ins(1,4,5)P$_3$-evoked calcium release contributes to light adaptation in *Limulus* ventral photoreceptors has not been estimated.

We attempted to reexamine this question by determining the effect of heparin

injection on $[Ca^{2+}]_i$ transients evoked by bright flashes. We used the metallochromic indicator dye, arsenazo III, to measure $[Ca^{2+}]_i$ transients in ventral photoreceptors; arsenazo III has been used previously in ventral photoreceptors to monitor $[Ca^{2+}]_i$ transients in response to light stimulation (Brown et al., 1977; Maaz and Stieve, 1980; Harary and Brown, 1984). Arsenazo III was injected intracellularly until a slight broadening of the transient portion of the receptor potential to bright flashes (4 s,
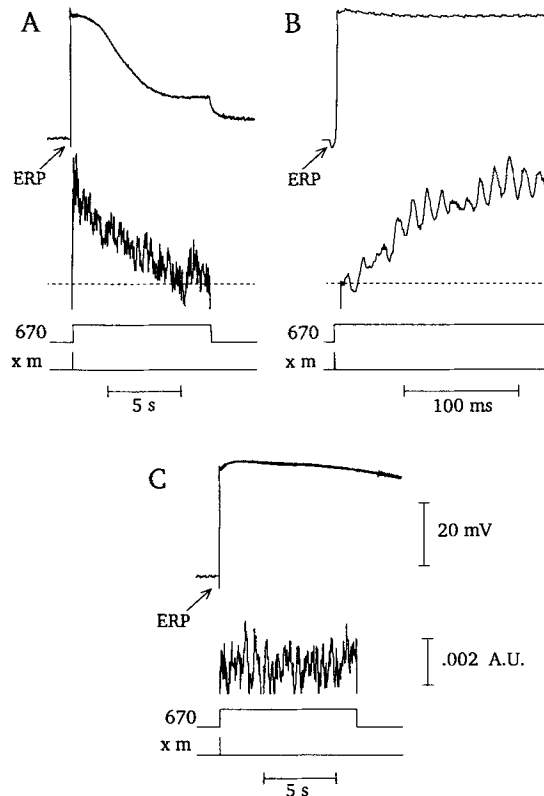
FIGURE 2. Heparin injection attenuates the light-induced increase in $[Ca^{2+}]_i$ in *Limulus* ventral photoreceptors. Photoreceptors were loaded with several injections of 25 mM arsenazo III and the electrode was withdrawn. Arsenazo III–loaded photoreceptors were re-impaled with a microelectrode containing 100 mg/ml heparin. (*A*) Receptor potentials and changes in absorbance of intracellular arsenazo III. A brief (1 ms) actinic flash from a xenon flashlamp (model 35-S flash tube, model 238 Strobex power supply; Chadwick-Helmuth Co., El Monte, CA), sufficiently intense to evoke a prominent early receptor potential (*arrows*), was given at the beginning of the measurement of absorbance at 670 nm (10-s duration). The traces labeled xm and 670 indicate the timing of the flash from the xenon flashlamp and the absorbance measurement beam, respectively. (*B*) Records from *A* plotted at a higher time resolution. Horizontal scale bar: 100 ms. (*C*) After the responses in *A* were recorded, 15 intracellular injections of heparin solution were made. The intracellular heparin made the light-induced change in absorbance of arsenazo III undetectable and made the receptor potential approximately square. All parts of the figure were taken from a single cell.

one every 60 s) was observed (usually after 5–10 injections). This physiological criterion was useful to predict when the intracellular concentration of arsenazo III was sufficient to produce a measurable absorbance change in response to light. Records from a cell injected with arsenazo III to this physiological criterion are shown in Fig. 2. A bright flash (from a xenon flash lamp) was delivered concurrently with the onset of a 10-s measurement light (at 670 nm). The receptor potential elicited by this

combined illumination had a transient phase that declined to a plateau; concurrently, the absorbance due to intracellular arsenazo III had a transient absorbance increase at 670 nm (Fig. 2, A and B). This transient absorbance increase was delayed with respect to the onset of the receptor potential and decayed to near baseline over the 10-s duration of the measurement light. If more arsenazo III was injected, the receptor potentials elicited by the combined illumination tended to become square, presumably because the intracellular arsenazo III strongly buffered $[Ca^{2+}]_i$. In this latter condition, the concurrent absorbance change at 670 nm typically was not transient but increased continuously or rose to an apparent plateau value as has been described previously (Brown et al., 1977).

Fig. 2 C illustrates the effect of heparin injection on the receptor potential and the light-induced increase in $[Ca^{2+}]_i$. After the responses shown in Fig. 2 A were recorded, the cell was injected with heparin solution. The light-induced change in absorbance of intracellular arsenazo III was undetectable after the heparin injections and the receptor potential (recorded concurrently) became approximately square. Similar results were obtained in two additional cells. These results suggest that an Ins(1,4,5)P$_3$-induced increase in $[Ca^{2+}]_i$ may account entirely for the light-induced increase in $[Ca^{2+}]_i$ and that this mechanism is largely responsible for initiating light adaptation.

Heparin Injection Blocks Bursts of Depolarizations Induced by Ins(1,4,5)P₃ Injection

Intracellular injection of Ins(1,4,5)P$_3$ into *Limulus* ventral photoreceptors often induces bursts of discrete depolarizations (Brown et al., 1984; Fein et al., 1984). These bursts of discrete depolarizations have been found to be accompanied by bursts of individual increases in $[Ca^{2+}]_i$ (Corson and Fein, 1987). The cell body of an isolated ventral photoreceptor is typically divided into two lobes: a light-sensitive rhabdomeral (R) lobe and a light-insensitive arhabdomeral (A) lobe (Calman and Chamberlain, 1982; Stern, Chinn, Bacigalupo, and Lisman, 1982). Ins(1,4,5)P$_3$-induced calcium release and light-induced calcium release from intracellular stores are colocalized in the R lobe (Payne and Fein, 1987; Payne, Walz, Levy, and Fein, 1988). Injections of 1 mM Ins(1,4,5)P$_3$ into the A lobe induce bursts of discrete depolarizations after a variable latency that may continue for several seconds after the injection (Payne and Potter, 1991).

We examined the effects of heparin injection on the discrete depolarizations induced by injection of Ins(1,4,5)P$_3$ and on the responses to dim lights. Dark-adapted photoreceptors were impaled with two microelectrodes, one containing Ins(1,4,5)P$_3$ and the other containing heparin. Only photoreceptors that produced a burst of discrete depolarizations after Ins(1,4,5)P$_3$ injection were considered in the following analysis. We recorded responses elicited by a series of flashes of progressively increasing intensity and by Ins(1,4,5)P$_3$ injections both before and after heparin injection (Fig. 3). Before heparin injection, Ins(1,4,5)P$_3$ injection induced bursts of discrete depolarizations resembling the response to a dim light. After heparin injection, a response to Ins(1,4,5)P$_3$ injection was largely absent. The effects of heparin injection on the light response were threefold: (a) individual quantum bumps in response to dim lights were greatly reduced in amplitude; (b) the amplitude of the transient phase of receptor potentials elicited by bright flashes was reduced, whereas

(c) the amplitude of the plateau was increased. Additional injections of heparin often produced a receptor potential that was approximately square (see Figs. 2 C and 9 B). Similar results were obtained in four additional cells. These findings differ from those of Frank and Fein (1991), who found that injections of 1 mg/ml heparin reduced only the amplitude of the transient phase of the response to bright flashes and had little effect on the amplitude of the plateau response.
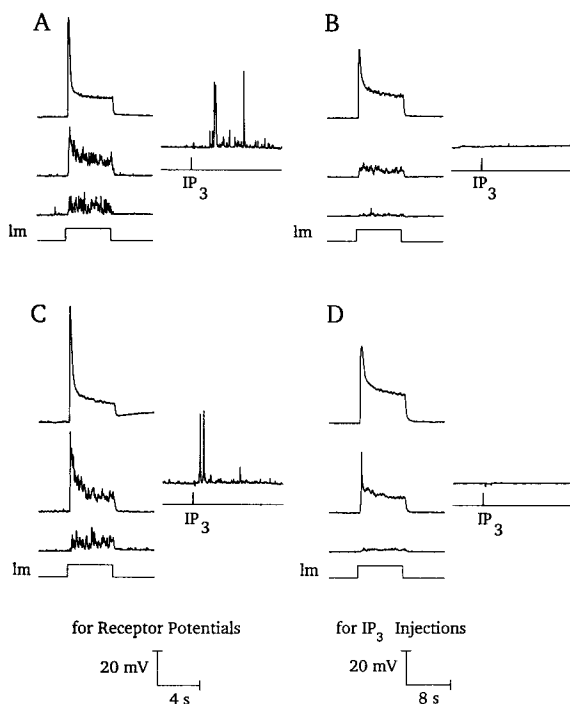

FIGURE 3. Effects of heparin injection on both receptor potentials and discrete depolarizations induced by Ins(1,4,5)P₃ injection. Dark-adapted photoreceptors were impaled with two microelectrodes, one containing 1 mM Ins(1,4,5)P₃ and one containing 100 mg/ml heparin. Data were recorded from two cells before (A and C) and after (B and D) eight injections of heparin solution. The responses to three flash intensities (4-s duration; 6.0, 4.8, and 2.4 log units attenuation) and a single Ins(1,4,5)P₃ injection are shown. Flash duration was 4 s. lm, light monitor. The time scale is two times slower in Ins(1,4,5)P₃ injection records than in light response records. Note that after heparin injection the responses to Ins(1,4,5)P₃ are largely absent and that the amplitude of the transient phase of the receptor potential is reduced but that of the plateau is increased.

Injection of BAPTA or Heparin Produces Similar Effects on the Stimulus–Response Relation for the Light-induced Current

Intracellular pressure injection of the calcium chelator EGTA greatly slows the rate of rise and reduces the peak amplitude of the light-induced current in response to a brief flash (Brown and Lisman, 1975; Payne and Fein, 1986; Payne et al., 1986). These effects presumably result from stabilization of $[Ca^{2+}]_i$. Because the light-induced increase in $[Ca^{2+}]_i$ in *Limulus* ventral photoreceptors is attenuated by intracellular injection of heparin (Fig. 2), heparin injection might be expected to produce effects on the kinetics and amplitude of the light-induced current similar to those observed after injection of a calcium buffer. Frank and Fein (1991) observed

that intracellular pressure injection of BAPTA or heparin reduced the peak amplitude of the light-induced current in response to a brief flash similar to the effect of EGTA; however, they did not report that either compound slowed the rate of rise of the light-induced current. We reexamined this question by comparing the effects of intracellular injection of BAPTA or heparin on light-induced currents in response to brief flashes. Fig. 4 *A* contains representative records of light-induced currents from the linear range of the stimulus–response relation recorded from a single cell before
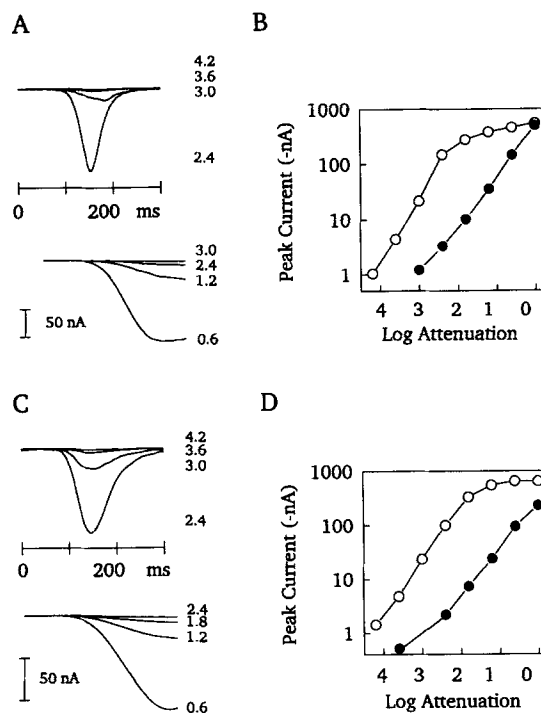

FIGURE 4. Changes in the stimulus–response relation for brief flashes are similar in the presence of a high intracellular concentration of heparin or BAPTA. Voltage-clamped responses to a series of progressively brighter flashes (20 ms, one every 60 s) were recorded before and after injection of either BAPTA or heparin. Light-induced currents that have similar amplitudes before and after injections are plotted for comparison (log attenuation of stimuli are given to the right of each light-induced current in *A* and *C*). (*A*) Voltage-clamp currents before (*top*) and after (*bottom*) injection of BAPTA (43 injections of 300 mM BAPTA during 30 min). (*B*) Peak amplitudes of light-induced currents are plotted before (*open circles*) and after (*closed circles*)

intracellular injection of BAPTA. (*C*) Voltage-clamp currents before (*top*) and after (*bottom*) injection of heparin (43 injections of 100 mg/ml heparin during 20 min). (*D*) Peak amplitudes of light-induced currents are plotted. The rate of rise of the light-induced current was dramatically slowed in the presence of a high intracellular concentration of either BAPTA or heparin. The stimulus–response relation was shifted along the intensity axis by ~2 log units in the presence of a high intracellular concentration of either BAPTA or heparin. Vertical scale bar: 200 nA.

and after BAPTA was injected to a high intracellular concentration. The rate of rise of the current induced by brief flashes was greatly reduced after BAPTA injection, similar to the effect previously observed after injection of EGTA. In addition, the light intensity required to evoke a particular amplitude current after injection of BAPTA was nearly 2 log units brighter than that required to evoke a similar amplitude current in the control condition. The stimulus–response relation for the peak amplitude of the light-induced current was shifted to brighter light intensities

by an average of 1.6 log units (average from six cells, SD = 0.8 log units) after injection of BAPTA. Fig. 4 C contains representative records of light-induced currents from the linear range of the stimulus–response relation recorded from a single cell before and after injection of heparin to a high intracellular concentration. The rate of rise of the light-induced current was slower after heparin injection, similar to the effect after BAPTA injection. In addition, the light intensity required to evoke a particular amplitude current after heparin injection was nearly 2 log units brighter than that required to evoke a similar amplitude current in the control condition. The stimulus–response relation for the peak amplitude of light-induced current was shifted to brighter light intensities by an average of 2.8 log units (average from four cells, SD = 0.8 log units) after heparin injection. These results suggest that both BAPTA and heparin reduce the rate of rise of the light-induced current and reduce the sensitivity by attenuating the light-induced increase in $[Ca^{2+}]_i$.

Injection of Neomycin or Spermine Reduces the Amplitude and Alters the Kinetics of the Light-induced Current

The light-stimulated activity of phospholipase C that hydrolyzes PtdInsP$_2$ has been measured in invertebrate photoreceptors by the appearance of Ins(1,4,5)P$_3$ (Brown et al., 1984; Szuts et al., 1986; Brown et al., 1987; Devary et al., 1987; Baer and Saibil, 1988; Wood, Szuts, and Fein, 1989). If phospholipase C activation is involved in phototransduction only through Ins(1,4,5)P$_3$-induced calcium release, then intracellular injection of inhibitors of phospholipase C activity would be expected to produce effects on phototransduction similar to those observed after intracellular injection of heparin. The polyamines, spermine and neomycin, have been demonstrated to inhibit phospholipase C activity in other preparations (neomycin: Schibeci and Schacht, 1977; Downes and Michell, 1981; spermine: Wojcikiewicz and Fain, 1988). The mechanism of this effect is probably not due to a direct interaction with the phospholipase C enzyme, but instead is due to the ability of these compounds to bind tightly to the phospholipase C substrate, PtdInsP$_2$ (neomycin: Schact, 1978; spermine: Meers, Hong, Bentz, and Papahadjopoulos, 1986; Tadolini and Varani, 1986).

To assay the effects of neomycin or spermine on phototransduction, we recorded the light-induced current in response to brief flashes before and after intracellular injection of one of the polyamines. The injection micropipette was filled with a 100-mM solution of either neomycin or spermine. The injections (one or two injections per cell) reduced the amplitude of the photocurrent (Fig. 5). A similar result has been reported previously in ventral photoreceptors after injection of neomycin (Frank and Fein, 1991). Fig. 5 also shows that an injection of neomycin or spermine solution produced changes in the waveform of the light-induced current similar to those observed after heparin injection; the time course of the rising phase of the light-induced current was substantially altered, while the falling phase remained relatively intact. The effect of one or two injections of either compound on the amplitude of the light-induced current was partially prevented by prior injection of BAPTA to a high intracellular concentration. Data from these experiments are summarized in Table II. These results are similar to those obtained after intracellular injection of 100 mg/ml heparin (Table I). The amplitude of the light-induced current was slightly reduced and the falling phase of the response was prolonged after multiple (≥ 10) injections of either spermine or neomycin in a cell previously loaded

A

100 ms

B

500 ms

15 nA

15 nA

1 m

1 m
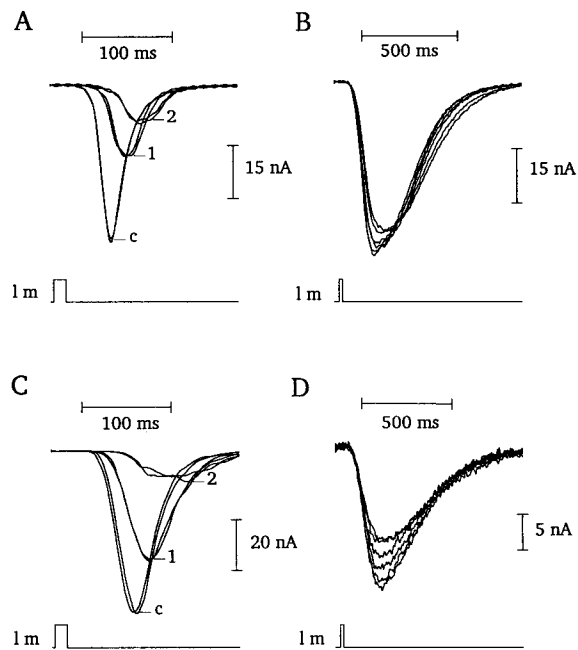
C

100 ms

D

500 ms

20 nA

5 nA

1 m

1 m

FIGURE 5. Effects of spermine and neomycin injection on light-induced currents. (*A*) Six consecutive light-induced currents in response to 20-ms flashes (one every 30 s, 3.0 log attenuation): two before injection, two after one injection of 100 mM spermine, and two after a second injection of spermine solution. (*B*) Six consecutive light-induced currents in response to 20-ms flashes (one every 30 s, 1.8 log attenuation) before and after injections of 100 mM spermine (three injections each after the second and fourth current record) in a cell previously injected to a high intracellular concentration of BAPTA. Before impalement with a microelectrode containing spermine solution, the cell was injected with BAPTA as described in Fig. 1. (*C*) Six consecutive light-induced currents in response to 20-ms flashes (one every 30 s, 3.0 log attenuation): two before injection, two after one injection of 100 mM neomycin, and two after a second injection of neomycin solution. (*D*) Six consecutive light-induced currents in response to 20-ms flashes (one every 30 s, 1.8 log attenuation) from a cell previously injected with BAPTA before and after injections of 100 mM neomycin solution (two each after the second and fourth current records). Vertical scale bars are 15 nA (*A*), 15 nA (*B*), 20 nA (*C*), and 5 nA (*D*). *A–D* were each taken from a different cell. Horizontal scale bar: 100 ms for *A* and *C*; 500 ms for *B* and *D*.

with BAPTA (data not shown). Further injections of neomycin or spermine solution, beyond that required to produce the effects illustrated in Fig. 5, produced a dramatic slowing of the time course of the light response. Fig. 6 shows records of receptor potentials in response to 4-s flashes recorded from five different cells that were

TABLE II

Effect of Neomycin and Spermine on the Amplitude of the Light-induced Current

Substance injected	% Control amplitude Mean ± SD (n)
Neomycin	56.1 ± 16.2 (8)
Neomycin + BAPTA	82.9 ± 11.3 (5)
Spermine	33.0 ± 13.0 (8)
Spermine + BAPTA	77.0 ± 13.0 (5)

The concentration of polyamine in the injection solutions was 100 mM. For each cell, the response amplitude after injection was normalized by dividing by the response amplitude before injection (one or two visible injections). The mean normalized response amplitude after injection is tabulated as % control amplitude ± SD; number of cells tested (n).

injected with progressively more injection solution that contained either BAPTA (Fig. 6 A), heparin (Fig. 6 B), neomycin (Fig. 6 C), spermine (Fig. 6 D), or putrescine (Fig. 6 E). Both BAPTA and heparin, when injected to a high intracellular concentration, attenuated the transient-to-plateau transition of the receptor potential so that the waveform became approximately square. Although intracellular injection of BAPTA or heparin slowed the kinetics of the light-induced current in response to a brief flash (Fig. 5), the waveform of the response to longer flashes, viewed on the time scale of
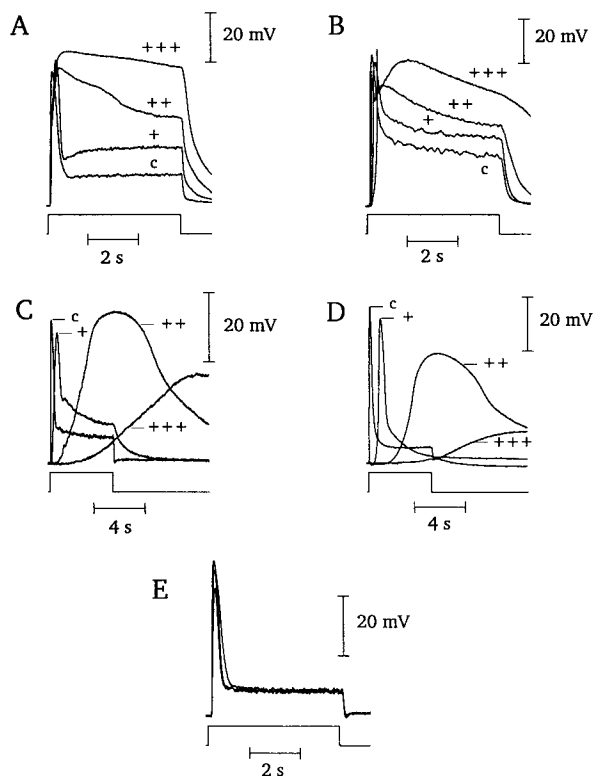

FIGURE 6. Changes in receptor potential waveforms induced by high intracellular concentrations of BAPTA, heparin, neomycin, spermine, and putrescine. Each part of the figure contains selected records of receptor potentials recorded after progressively more intracellular injection of each substance. All receptor potentials are in response to 4-s flashes (2.4 log attenuation). (A) Receptor potentials recorded from a cell injected with BAPTA (300 mM, 0–28 injections). (B) Receptor potentials recorded from a cell injected with heparin (100 mg/ml, 0–33 injections). (C) Receptor potentials recorded from a cell injected with neomycin (100 mM, 0–11 injections). (D) Receptor potentials recorded from a cell injected with spermine (250 mM, 0–24 injections). (E) Receptor potentials recorded from a cell injected with putrescine (500 mM, 0–20 injections). Vertical scale bars: 20 mV. The bottom trace of each group is a stimulus monitor; all flash durations were 4 s.

seconds, was not greatly prolonged (Fig. 6, A and B). In contrast, high intracellular concentrations of either neomycin or spermine greatly slowed the kinetics of the flash response to the point that the peak of the light response frequently occurred several seconds after the end of the stimulus. It is unlikely that the effects of neomycin or spermine are solely a consequence of their high charge density; injection of 500 mM putrescine (Fig. 6 E), 200 mM spermidine (three cells), or a 200-mM solution of the dipeptide Arg-Lys (three cells) did not produce similar changes in the waveform of the light response.
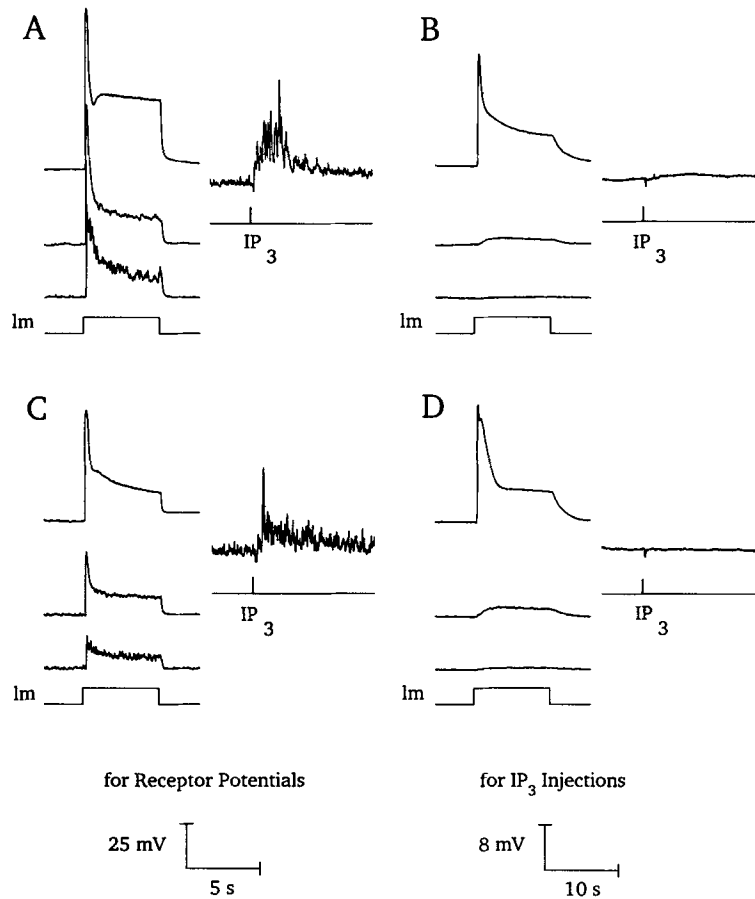
FIGURE 7. Intracellular injection of neomycin or spermine desensitizes ventral photoreceptors to light or injected $Ins(1,4,5)P_3$. (A) Receptor potentials (*left*) to progressively brighter 5-s flashes (6.0, 4.8, and 2.4 log attenuation) and the response to injection of 1 mM $Ins(1,4,5)P_3$ (*right*) into the A lobe. (B) Receptor potentials (*left*) and the response to injection of 1 mM $Ins(1,4,5)P_3$ (*right*) after intracellular injection of neomycin (100 mM, 4 injections). Neomycin desensitized the cell to both light and injection of $Ins(1,4,5)P_3$. Data in A and B are from the same cell. (C) Receptor potentials (*left*) to progressively brighter 5-s flashes (6.0, 4.8, and 2.4 log attenuation) and the response to injection of 1 mM $Ins(1,4,5)P_3$ (*right*) into the A lobe recorded. (D) Receptor potentials (*left*) and the response to injection of $Ins(1,4,5)P_3$ (*right*) after injection of a spermine (250 mM, 11 injections). Similar to the effect of neomycin, spermine desensitized the cell to both light stimulation and injection of $Ins(1,4,5)P_3$. Data in C and D are from the same cell. Vertical scale bar: 25 mV for receptor potentials and 8 mV for the responses to injections of $Ins(1,4,5)P_3$. Horizontal scale bar: 5 s for receptor potentials and 10 s for $Ins(1,4,5)P_3$ injections.

We also examined the effects of intracellular injection of either spermine or neomycin on the waveform of the response to injected $Ins(1,4,5)P_3$. If the effects of these compounds are simply a consequence of inhibition of phospholipase C activity, then the response to injected $Ins(1,4,5)P_3$ might be expected to be largely intact. To test this prediction, we recorded voltage changes in response to light and to

injections of Ins(1,4,5)P$_3$ (as in Fig. 3) before and after intracellular injections of either neomycin (Fig. 7, A and B) or spermine (Fig. 7, C and D). Both compounds desensitized the recorded responses to light stimulation and Ins(1,4,5)P$_3$ injection. Similar results were obtained from two additional cells injected with neomycin and two additional cells injected with spermine. Previously, Frank and Fein (1991) reported that injections out of a pipette filled with a 5.5-mM solution of neomycin reduced the sensitivity of photoreceptors to transient light stimulation but not to Ins(1,4,5)P$_3$ injected into the R lobe of the cell. Why that study and ours differ with regard to the effect of neomycin on the sensitivity of photoreceptors to Ins(1,4,5)P$_3$ injections is not clear.

DISCUSSION

Effects of Heparin Injections on the Light-induced Current

Our observations suggest that intracellularly injected heparin tends to block calcium release from Ins(1,4,5)P$_3$-sensitive stores in *Limulus* photoreceptors. First, we have demonstrated that heparin, injected to a high intracellular concentration, can attenuate the increase in [Ca^{2+}]$_i$ stimulated by a bright flash. Second, we and others (Frank and Fein, 1991) have observed that heparin attenuates depolarizations induced by intracellular injections of Ins(1,4,5)P$_3$. This result is consistent with heparin acting to attenuate Ins(1,4,5)P$_3$-induced calcium release because an increase in [Ca^{2+}]$_i$ is probably an obligatory step in the mechanism through which intracellularly injected Ins(1,4,5)P$_3$ leads to the depolarization of photoreceptors (Rubin and Brown, 1985; Payne et al., 1986).

An alternative explanation for the mechanism of the observed effects of heparin is that the calcium binding capacity of injected heparin largely buffers changes in [Ca^{2+}]$_i$. This explanation is not probable because heparin has a relatively low affinity for calcium. In the presence of 300 mM monovalent salt, the apparent K_D of heparin for calcium is estimated to be ~80,000 μM (extrapolated from data in Mattai and Kwak, 1981). In similar ionic conditions BAPTA has an apparent K_D for calcium of 0.59 μM (Pethig, Kuhn, Payne, Adler, Chen, and Jaffe, 1989). An estimate from this comparison suggests that ~130,000 times more heparin would be required to buffer intracellular calcium to the same extent as a given amount of BAPTA. The equivalent concentration of heparin in our injection solution was 56 mM (calculated using an equivalent weight of heparin = 178 g; Mattai and Kwak, 1981). A similar number of injections of this heparin solution or a 300-mM solution of BAPTA were required to produce noticeable changes in the waveform of the light response. Therefore, the effects we observed after heparin injection are unlikely to be attributable to the calcium-binding properties of heparin.

The effects we observe after intracellular injection of heparin indicate that Ins(1,4,5)P$_3$-induced calcium release participates in the mechanism of excitation of ventral photoreceptors. After heparin was injected into a photoreceptor, the current induced by a brief flash was reduced in amplitude relative to the amplitude before injection; this effect was not seen in cells injected with BAPTA before heparin injection. These results suggest that the Ins(1,4,5)P$_3$-induced rise in [Ca^{2+}]$_i$ enhances the amplification of the phototransduction cascade in a dark-adapted photoreceptor.

Moreover, $[Ca^{2+}]$ may itself enhance the effects of an increase in $Ins(1,4,5)P_3$; $[Ca^{2+}]$ has been found to act as a "coagonist" in some preparations. However, $Ins(1,4,5)P_3$-mediated actions may still occur at very low concentrations of $[Ca^{2+}]$ (Bezprozvanny, Watras, and Ehrlich, 1991; Finch, Turner, and Goldin, 1991; Iino and Endo, 1992). Bolsover and Brown (1985) and Faddis and Brown (1992a, b) have reported previously that an increase in $[Ca^{2+}]_i$ enhances excitation in ventral photoreceptors in some circumstances. In addition, pressure injection of calcium solutions can excite ventral photoreceptors (Payne et al., 1986). Calcium ions probably do not directly gate the light-activated conductance because calcium ions do not open channels in excised patches of membrane that contained light-activated channels before excision (Bacigalupo, Johnson, Vergara, and Lisman, 1991). We suggest that the $Ins(1,4,5)P_3$-induced rise in $[Ca^{2+}]_i$ may accelerate one or more steps in the cascade of reactions that produces the ligand for the light-activated channel.

Our results for the effects of heparin on the response to a brief flash differ from those previously reported in ventral photoreceptors (Frank and Fein, 1991). These authors reported that injections of a 1 mg/ml heparin solution into ventral photoreceptors reduced the amplitude of the responses to brief flashes; however, we were unable to observe a significant effect from an injection of a heparin solution containing < 100 mg/ml. In addition, Frank and Fein (1991) reported that heparin injections reduced the amplitude of the response to a brief flash but did not alter its time course; however, we have found that an injection of a 100 mg/ml heparin solution produces marked changes in the time course of the current induced by a brief flash in addition to a reduction in the amplitude of the response. When injected to a high intracellular concentration, heparin markedly slows the waveform of the light-induced current. This effect is very similar to that observed after BAPTA (this study) or EGTA (Lisman and Brown, 1975; Payne et al., 1986; Payne and Fein, 1986) is injected to a high intracellular concentration.

Our results for the effects of heparin on the response to a long flash differ from those previously reported in ventral photoreceptors (Frank and Fein, 1991). These authors reported that heparin injections reduced the amplitude of the transient component of the response to a long flash but had little effect on the plateau component of the response; however, we have found that a high intracellular concentration of heparin or BAPTA reduces the transient-to-plateau transition of the receptor potential in response to a long flash of light. As a result, the waveform of the light response becomes approximately square, similar to the effect observed after intracellular injections of EGTA (Lisman and Brown, 1975). The effect of EGTA on the waveform of the light response, together with the observations that light induces an increase in $[Ca^{2+}]_i$, have been interpreted to suggest that calcium ions mediate the mechanism of light adaptation in ventral photoreceptors (Brown and Blinks, 1974; Lisman and Brown, 1975; Brown et al., 1977; Harary and Brown, 1984; Payne and Fein, 1987). The observation that intracellular injection of heparin attenuated the increase in $[Ca^{2+}]_i$ induced by a flash that activated most of the available rhodopsin in a cell suggests that $Ins(1,4,5)P_3$-induced calcium release is the principal mechanism through which light increases $[Ca^{2+}]_i$ within *Limulus* ventral photoreceptor cells. This conclusion cannot be generalized to all invertebrate photoreceptors; calcium entry through the light-activated conductance may be the principal mechanism underlying the light-induced increase in $[Ca^{2+}]_i$ for *Drosophila* (Ranganathan, Harris, Stevens,

and Zuker, 1991) and *Balanus* (Brown and Blinks, 1974) photoreceptors. Whether Ins(1,4,5)P$_3$-induced calcium release can account entirely for the light-induced increase in [Ca^{2+}]$_i$ in *Limulus* ventral photoreceptors cannot be concluded from our data because the possibility that Ins(1,4,5)P$_3$-induced calcium release may "trigger" other calcium release processes has not been excluded.

Our results support the hypothesis that Ins(1,4,5)P$_3$-induced calcium release participates in two apparently antagonistic processes in *Limulus* ventral photoreceptors: a mechanism by which an Ins(1,4,5)P$_3$-induced increase in [Ca^{2+}]$_i$ enhances the amplification of the phototransduction cascade, and a mechanism by which an Ins(1,4,5)P$_3$-induced increase in [Ca^{2+}]$_i$ mediates the mechanism of light adaptation, a process that reduces the amplification of the phototransduction cascade (Lisman and Brown, 1975). The implication from the apparent coexistence of these two antagonistic processes initiated by Ins(1,4,5)P$_3$ is that the kinetics or the concentration dependence of the two mechanisms is dissimilar.

Effects of Polyamine Injections on the Light Response

We investigated whether Ins(1,4,5)P$_3$-induced calcium release was the only mechanism through which phospholipase C activation participates in excitation of ventral photoreceptors by a comparison of the effects of intracellular injections of heparin with those of intracellular injections of the polyamines, neomycin and spermine. The validity of this comparison depends on the specificity of the polyamines for inhibiting phospholipase C activity (neomycin: Schibeci and Schacht, 1977; Downes and Michell, 1981; spermine: Wojcikiewicz and Fain, 1988) versus interactions with other components of the phototransduction cascade. We found that intracellular injection of either neomycin or spermine to low intracellular concentrations produced effects on the kinetics and amplitude of the light-activated current similar to those found after intracellular injection of heparin; these results suggest that the dominant effect of neomycin or spermine on phototransduction is inhibition of Ins(1,4,5)P$_3$ release from PtdInsP$_2$. These effects of neomycin or spermine were not produced by intracellular injections of other polycationic compounds (putrescine, spermidine, Arg-Lys dipeptide).

In contrast to the effects of low intracellular concentrations of spermine or neomycin, after photoreceptors are injected to a high intracellular concentration of either compound, the waveform of the response to long flashes is dramatically prolonged and distorted and the response to injected Ins(1,4,5)P$_3$ is desensitized. These effects may reflect interactions by neomycin or spermine with other components of the phototransduction cascade in addition to phospholipase C. Polyamines are known to interact with a variety of intracellular targets (reviewed in Marton and Morris, 1987).

An alternative explanation is that some of these effects of high intracellular concentrations of polyamines might result from a profound inhibition of phospholipase C. The hydrolysis of PtdInsP$_2$ by phospholipase C will produce both InsP$_3$ and diacylglycerol. Diacylglycerol is known to activate many of the protein kinase C (PKC) family of enzymes (Nishizuka, 1986). Thus, phospholipase C inhibition by polyamines might reduce diacylglycerol production and subsequently reduce activation of PKC. Whether a PKC activated by diacylglycerol participates in excitation of *Limulus* photoreceptors is unknown. Recently, a *Drosophila* mutation has been identified that

lacks a functional photoreceptor-specific PKC gene (Smith, Ranganathan, Hardy, Marx, Tsuchida, and Zuker, 1991); in photoreceptors possessing this genetic defect, the falling phase of the light-induced current was slowed relative to wild-type photoreceptors, but the rising phase was unchanged (Ranganathan et al., 1991). These authors suggest that PKC activity may regulate the kinetics of the light-induced current and participate in the mechanism of rapid desensitization.

It has been proposed that a light-activated phospholipase C that hydrolyzes phosphoinositides is an obligatory step in the transduction cascade in fly photoreceptors (e.g., Minke and Selinger, 1992). The foundations of that proposal are that strong alleles of *norpA* mutants lack receptor potentials (Pak, Ostroy, Deland, and Wu, 1976) and light-activated phospholipase C (Inoue, Yoshioka, and Hotta, 1985), and that the *norpA* gene product is highly homologous with a phospholipase C (Bloomquist, Shortridge, Schneuwly, Perdew, Montell, Steller, Rubin, and Pak, 1988). The proposal that the production of $InsP_3$ by a phospholipase C is obligatory in *Limulus* photoreceptors is doubtful for the following two reasons. First, introduction of calcium-sequestering agents (EGTA, BAPTA, arsenazo III, etc.) greatly attenuates responses to intracellular injection of $InsP_3$ but does not interrupt the light responses (Rubin and Brown, 1985; Payne et al., 1986). Second, intracellular injection of heparin greatly attenuates the light-induced increase in $[Ca^{2+}]_i$, whereas the light response is preserved. However, these data do not exclude the possibility that diacylglycerol, also produced by phospholipase C hydrolysis of $PtdInsP_2$, may participate in the *Limulus* phototransduction cascade.

Our findings suggest that phospholipase C activation does participate in both excitation and light adaptation in *Limulus* photoreceptors through mechanisms initiated by the $Ins(1,4,5)P_3$-induced increase in $[Ca^{2+}]_i$. Our data further suggest that in the absence of an $Ins(1,4,5)P_3$-induced increase in $[Ca^{2+}]_i$ excitation can proceed, but that the gain and the speed of the transduction cascade are reduced. In contrast to these effects on excitation, our data suggest that light adaptation is largely attenuated in the absence of the $Ins(1,4,5)P_3$-induced increase in $[Ca^{2+}]_i$. If this is so, $Ins(1,4,5)P_3$ may be properly considered an intracellular second messenger of light adaptation in ventral photoreceptors. Because our findings for the effects of neomycin or spermine on phototransduction are dependent on concentration, we cannot rigorously exclude the possibility that phospholipase C activation participates in phototransduction other than through the $Ins(1,4,5)P_3$-induced increase in $[Ca^{2+}]_i$.

REFERENCES

Bacigalupo, J., E. Johnson, P. Robinson, and J. E. Lisman. 1990. Second messengers in invertebrate phototransduction. *In* Transduction in Biological Systems. C. Hidalgo, J. Bacigalupo, E. Jaimovich, and J. Vergara, editors. Plenum Publishing Corp., New York. 27–45.

Bacigalupo, J., E. C. Johnson, C. Vergara, and J. E. Lisman. 1991. Light-dependent channels from excised patches of *Limulus* ventral photoreceptors are opened by cGMP. *Proceedings of the National Academy of Sciences, USA.* 88:7938–7942.

Baer, K. M., and H. R. Saibil. 1988. Light- and GTP-activated hydrolysis of phosphatidylinositol bisphosphate in squid photoreceptor membranes. *Journal of Biological Chemistry.* 263:17–20.

Bezprozvanny, I., J. Watras, and B. E. Ehrlich. 1991. Bell-shaped calcium-response curves of Ins(1,4,5)P3- and calcium-gated channels from endoplasmic reticulum of cerebellum. *Nature.* 351:752–754.

Bloomquist, B. T., R. D. Shortridge, S. Schneuwly, M. Perdew, C. Montell, H. Steller, G. Rubin, and W. L. Pak. 1988. Isolation of a putative phospholipase C gene of *Drosophila, norpA*, and its role in phototransduction. *Cell.* 54:723–733.

Bolsover, S. R., and J. E. Brown. 1985. Calcium ion, an intracellular messenger of light adaptation, also participates in excitation of *Limulus* ventral photoreceptor cells. *Journal of Physiology.* 364:381–393.

Brown, J. E. 1986. Calcium and light adaptation in invertebrate photoreceptors. *In* The Molecular Mechanism of Photoreception. H. Stieve, editor. Dahlem Konferenzen, Berlin. 231–240.

Brown, J. E., and J. R. Blinks. 1974. Changes in intracellular free calcium concentration during illumination of invertebrate photoreceptors. Detection with aequorin. *Journal of General Physiology,* 64:643–665.

Brown, J. E., P. K. Brown, and L. H. Pinto. 1977. Detection of light-induced changes of intracellular ionized calcium concentration in *Limulus* ventral photoreceptors using arsenazo III. *Journal of Physiology.* 267:299–320.

Brown, J. E., H. H. Harary, and A. Waggoner. 1979. Isopotentiality and an optical determination of series resistance in *Limulus* ventral photoreceptors. *Journal of Physiology.* 296:357–372.

Brown, J. E., and J. E. Lisman. 1975. Intracellular Ca modulates sensitivity and time scale in *Limulus* ventral photoreceptors. *Nature.* 258:252–254.

Brown, J. E., and M. I. Mote. 1974. Ionic dependence of reversal voltage of the light response in *Limulus* ventral photoreceptors. *Journal of General Physiology.* 63:337–350.

Brown, J. E., and L. J. Rubin. 1984. A direct demonstration that inositol-trisphosphate induces an increase in intracellular calcium in *Limulus* photoreceptors. *Biochemical and Biophysical Research Communications.* 125:1137–1142.

Brown, J. E., L. J. Rubin, A. J. Ghalayini, A. P. Tarver, R. F. Irvine, M. J. Berridge, and R. E. Anderson. 1984. Evidence that myo-inositol polyphosphate may be a messenger for visual excitation in *Limulus* photoreceptors. *Nature.* 311:160–163.

Brown, J. E., D. C. Watkins, and C. C. Malbon. 1987. Light-induced changes in the content of inositol phosphates in squid (*Loligo pealei*) retina. *Biochemical Journal.* 247:293–297.

Calman, B., and S. Chamberlain. 1982. Distinct lobes of *Limulus* ventral photoreceptors. II. Structure and ultrastructure. *Journal of General Physiology.* 80:839–862.

Clark, A. W., R. Millecchia, and A. Mauro. 1969. The ventral photoreceptors of *Limulus*. I. The microanatomy. *Journal of General Physiology.* 54:289–309.

Coles, J. A., and J. E. Brown. 1976. Effects of increased intracellular pH-buffering capacity on the light response of *Limulus* ventral photoreceptor. *Biochimica et Biophysica Acta.* 436:140–153.

Corson, D. W., and A. Fein. 1983. Quantitative pressure injection of picoliter volumes into *Limulus* ventral photoreceptors. *Biophysical Journal.* 44:299–304.

Corson, D. W., and A. Fein. 1987. Inositol 1,4,5 trisphosphate induces bursts of calcium release inside *Limulus* ventral photoreceptors. *Brain Research.* 423:343–346.

Cullen, P. J., G. G. Comerford, and A. P. Dawson. 1988. Heparin inhibits the inositol 1,4,5-trisphosphate-induced Ca^{2+} release from rat liver microsomes. *FEBS Letters.* 228:57–59.

Devary, O., O. Heichal, A. Blumenfeld, D. Cassel, E. Suss, S. Barash, C. T. Rubinstein, B. Minke, and Z. Selinger. 1987. Coupling of photoexcited rhodopsin to inositol phospholipid hydrolysis in fly photoreceptors. *Proceedings of the National Academy of Sciences, USA.* 84:6939–6943.

Downes, C. P., and R. H. Michell. 1981. The polyphosphoinositide phosphodiesterase of erythrocyte membranes. *Biochemical Journal.* 198:133–140.

Faddis, M. N. 1992. Studies on the mechanism of phototransduction in *Limulus* ventral photoreceptors. Ph.D. thesis. Washington University, St. Louis, MO. 182 pp.

Faddis, M. N., and J. E. Brown. 1992a. Flash photolysis of caged IP$_3$ in ventral photoreceptors of *Limulus. Biophysical Journal.* 61:A426. (Abstr.)

Faddis, M. N., and J. E. Brown. 1992b. Flash photolysis of caged compounds in *Limulus* ventral photoreceptors. *Journal of General Physiology.* 100:547–570.

Fein, A., R. Payne, D. W. Corson, M. J. Berridge, and R. F. Irvine. 1984. Photoreceptor excitation and adaptation by inositol 1,4,5-trisphosphate. *Nature.* 311:157–160.

Finch, E. A., T. J. Turner, and S. M. Goldin. 1991. Calcium as a coagonist of inositol 1,4,5-trisphosphate-induced calcium release. *Science.* 252:443–446.

Frank, T. M., and A. Fein. 1991. The role of the inositol phosphate cascade in visual excitation of invertebrate microvillar photoreceptors. *Journal of General Physiology.* 97:697–723.

Fuortes, M. G. F., and S. Yeandle. 1964. Probability of occurrence of discrete potential waves in the eye of *Limulus. Journal of General Physiology.* 47:443–463.

Harary, H. H., and J. E. Brown. 1984. Spatially nonuniform changes in intracellular calcium ion concentration. *Science.* 224:292–294.

Iino, M., and M. Endo. 1992. Calcium-dependent immediate feedback control of inositol 1,4,5-trisphosphate-induced Ca^{2+} release. *Nature.* 360:76–78.

Inoue, H., T. Yoshioka, and Y. Hotta. 1985. A genetic study of inositol trisphosphate involvement in phototransduction using *Drosophila* mutants. *Biochemical and Biophysical Research Communications.* 132:513–519.

Lisman, J. E., and J. E. Brown. 1975. Effects of intracellular injection of calcium buffers on light adaptation in *Limulus* ventral photoreceptors. *Journal of General Physiology.* 66:489–506.

Maaz, G., and H. Stieve. 1980. The correlation of the receptor potential with the light induced transient increase in intracellular calcium concentration measured by absorption change of arsenazo III injected into *Limulus* ventral nerve photoreceptor cell. *Biophysics of Structure and Mechanism.* 6:191–208.

Marton, L. J., and D. R. Morris. 1987. Molecular and cellular functions of the polyamines. *In* Inhibition of Polyamine Metabolism. P. P. McCann, A. E. Pegg, and A. Sjoerdsma, editors. Academic Press, New York. 79–105.

Mattai, J., and J. C. T. Kwak. 1981. Mg and Ca binding to heparin in the presence of added monovalent salt. *Biochimica et Biophysica Acta.* 677:303–312.

Meers, P., K. Hong, J. Bentz, and D. Papahadjopoulos. 1986. Spermine as a modulator of membrane fusion: interactions with acidic phospholipids. *Biochemistry.* 25:3109–3118.

Meyer, T., D. Holowka, and L. Stryer. 1988. Highly cooperative opening of calcium channels by inositol 1,4,5-trisphosphate. *Science.* 240:653–656.

Millecchia, R., and A. Mauro. 1969a. The ventral photoreceptor cells of *Limulus.* II. The basic photoresponse. *Journal of General Physiology.* 54:310–330.

Millecchia, R., and A. Mauro. 1969b. The ventral photoreceptor cells of *Limulus.* III. A voltage-clamp study. *Journal of General Physiology.* 54:331–351.

Minke, B., and Z. Selinger. 1992. The inositol-lipid pathway is necessary for light excitation in fly photoreceptors. *In* Sensory Transduction. D. P. Corey and S. D. Roper, editors. 202–217.

Nilsson, T., J. Zwiller, A. L. Boynton, and P. O. Berggren. 1988. Heparin inhibits IP3-induced Ca^{2+} release in permeabilized pancreatic beta-cells. *FEBS Letters.* 229:211–214.

Nishizuka, Y. 1986. Studies and perspectives of protein kinase C. *Science.* 233:305–312.

Pak, W. L., S. E. Ostroy, M. C. Deland, and C. F. Wu. 1976. Photoreceptor mutant of *Drosophila:* Is protein involved in intermediate steps of transduction? *Science.* 194:956–959.

Payne, R., D. W. Corson, and A. Fein. 1986. Pressure injection of calcium both excites and adapts *Limulus* ventral photoreceptors. *Journal of General Physiology.* 88:107–126.

Payne, R., D. W. Corson, A. Fein, and M. J. Berridge. 1986. Excitation and adaptation of *Limulus* ventral photoreceptors by inositol 1,4,5 trisphosphate result from a rise in intracellular calcium. *Journal of General Physiology.* 88:127–142.

Payne, R., and A. Fein. 1986. The initial response of *Limulus* ventral photoreceptors to bright flashes. *Journal of General Physiology.* 87:243–269.

Payne, R., and A. Fein. 1987. Inositol 1,4,5 trisphosphate releases calcium from specialized sites within *Limulus* photoreceptors. *Journal of Cell Biology.* 104:933–937.

Payne, R., and B. V. L. Potter. 1991. Injection of inositol trisphosphorothioate into *Limulus* ventral photoreceptors causes oscillations of free cytosolic calcium. *Journal of General Physiology.* 97:1165–1186.

Payne, R., B. Walz, S. Levy, and A. Fein. 1988. The localization of calcium release by inositol trisphosphate in *Limulus* photoreceptors and its control by negative feedback. *Philosophical Transactions of the Royal Society of London, Series B.* 320:359–379.

Pethig, R., M. Kuhn, R. Payne, E. Adler, T.-H. Chen, and L. F. Jaffe. 1989. On the dissociation constants of BAPTA-type calcium buffers. *Cell Calcium.* 10:491–498.

Ranganathan, R., G. L. Harris, C. F. Stevens, and C. S. Zuker. 1991. A *Drosophila* mutant defective in extracellular calcium-dependent photoreceptor deactivation and rapid desensitization. *Nature.* 354:230–232.

Rubin, L. J., and J. E. Brown. 1985. Intracellular injection of calcium buffers blocks IP3-induced but not light-induced electrical responses of *Limulus* ventral photoreceptors. *Biophysical Journal.* 47:38a. (Abstr.)

Schacht, J. 1978. Purification of polyphosphoinositides by chromatography on immobilized neomycin. *Journal of Lipid Research.* 19:1063–1067.

Schibeci, A., and J. Schacht. 1977. Action of neomycin on the metabolism of polyphosphoinositides in the guinea pig kidney. *Biochemical Pharmacology,* 26:1769–1774.

Schoenmakers, T. J. M., G. J. Visser, G. Flik, and A. P. R. Theuvenet. 1992. CHELATOR: an improved method for computing metal ion concentrations in physiological solutions. *BioTechniques.* 12:870–879.

Smith, D. P., R. Ranganathan, R. W. Hardy, J. Marx, T. Tsuchida, and C. S. Zuker. 1991. Photoreceptor deactivation and retinal degeneration mediated by a photoreceptor-specific protein kinase C. *Science.* 254:1478–1484.

Stern, J., K. Chinn, J. Bacigalupo, and J. E. Lisman. 1982. Distinct lobes of *Limulus* ventral photoreceptors. I. Functional and anatomical properties of lobes revealed by removal of glial cells. *Journal of General Physiology.* 80:825–837.

Szuts, E. Z., S. F. Wood, and A. Fein. 1986. Light stimulates the rapid formation of inositol trisphosphate in squid retinas. *Biochemical Journal.* 240:929–932.

Tadolini, B., and E. Varani. 1986. Interaction of spermine with polyphosphoinositides containing liposomes and myo-inositol 1,4,5-triphosphate. *Biochemical and Biophysical Research Communications.* 135:58–64.

Tones, M. A., M. D. Bootman, B. F. Higgins, D. A. Lane, G. F. Pay, and U. Lindahl. 1989. The effect of heparin on the inositol 1,4,5-trisphosphate receptor in rat liver microsomes. *FEBS Letters.* 252:105–108.

Wojcikiewicz, R. J. H., and J. N. Fain. 1988. Polyamines inhibit phospholipase C-catalyzed polyphosphoinositide hydrolysis. *Biochemical Journal.* 255:1015–1021.

Wood, S. F., E. Z. Szuts, and A. Fein. 1989. Inositol trisphosphate production in squid photoreceptors. *Journal of Biological Chemistry.* 264:12970–12976.

Worley, P. F., J. M. Baraban, S. Supattapone, V. S. Wilson, and S. H. Snyder. 1987. Characterization of inositol trisphosphate receptor binding in brain. *Journal of Biological Chemistry.* 262:12132–12136.