Flash Photolysis of Caged Compounds 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 Rapid concentration jumps of Ins(1,4,5)P_3 or ATP were made inside *Limulus* ventral photoreceptors by flash photolysis of the parent caged compounds. In intact ventral photoreceptors, the photolysis flash evokes a maximum amplitude light-activated current; therefore, a procedure was developed for uncoupling phototransduction by blocking two of the initial reactions in the cascade, rhodopsin excitation and G protein activation. Rhodopsin was inactivated by exposure to hydroxylamine and bright light. This procedure abolished the early receptor potential and reduced the quantum efficiency by 325 ± 90-fold (mean ± SD). G protein activation was blocked by injection of guanosine-5′-*O*-(2-thiodiphosphate) (GDPβS). GDPβS injection reduced the quantum efficiency by 1,881 ± 1,153-fold (mean ± SD). Together hydroxylamine exposure and GDPβS injection reduced the quantum efficiency by 870,000 ± 650,000-fold (mean ± SD). After the combined treatment, photoreceptors produced quantum bumps to light that was ~10^6 times brighter than the intensity that produced quantum bumps before treatment. Experiments were performed with caged compounds injected into photoreceptors in which phototransduction was largely uncoupled. Photolysis of one compound, *myo*-inositol 1,4,5-triphosphate $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester (caged IP_3), increased the voltage clamp current in response to the flashlamp by more than twofold without changing the latency of the response. The effect was not seen with photolysis of either adenosine-5′-triphosphate P^3-1-(2-nitrophenyl)ethyl ester (caged ATP) or caged IP_3 in cells preloaded with either heparin or (1,2-bis-(*o*-amino-phenoxy)ethane-*N*-*N*-*N*′-*N*′ tetraacetic acid tetrapotassium salt (BAPTA). The results suggest that photoreleased IP_3 releases calcium ions from intracellular stores and the resulting increase in $[Ca^{2+}]_i$ enhances the amplification of the phototransduction cascade.

INTRODUCTION

Photoreceptors of *Limulus* ventral eye are exquisitely sensitive to light when fully dark-adapted; the absorption of a single photon of light generates an easily measurable depolarization (a "quantum bump" or "discrete event") (Yeandle and Spiegler, 1973), which may be 10 or more mV in amplitude. Presently, the molecular

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

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details of the transduction mechanism underlying the process of excitation in invertebrate photoreceptors are poorly understood (reviewed in Bacigalupo, Johnson, Robinson, and Lisman, 1990). The experiments described in this paper attempt to apply a relatively new technology, flash photolysis of caged compounds, as a tool to study steps in the transduction cascade of *Limulus* ventral photoreceptors. The excellent temporal resolution inherent in this technique has been previously exploited to investigate the sequence of steps in the transduction cascade linking agonist binding to the opening of ion channels in guinea pig hepatocytes (Ogden, Capiod, Walker, and Trentham, 1990).

In previous studies, pressure injection of solutions from micropipettes has provided the best means for rapidly introducing compounds into *Limulus* ventral photoreceptors. Using this technique, several compounds have been found to depolarize ventral photoreceptors. Examples of such compounds include cGMP (Johnson, Robinson, and Lisman, 1986), Ins(1,4,5)P_3 (Brown, Rubin, Ghalayini, Tarver, Irvine, Berridge, and Anderson, 1984; Fein, Payne, Corson, Berridge, and Irvine, 1984) and calcium ion (Payne, Corson, and Fein, 1986*a*). However, the technique of intracellular pressure injection suffers from two technical problems that complicate the study of excitatory transduction. First, intracellular injection of a sufficient volume of any substance, regardless of its biological activity, may produce a depolarization due to damage (e.g., Bolsover and Brown, 1982). Second, pressure injection produces an intracellular concentration change with an unknown spatial and temporal distribution. The geometry of the cytosolic space is irregular and diverse in ventral photoreceptors (Clark, Millecchia, and Mauro, 1969; Calman and Chamberlain, 1982); therefore, the diffusion path within different cells may vary in tortuosity. This may explain the inconsistency of the highly focal responses reported for injection of some substances (e.g., Johnson et al., 1986).

Flash photolysis of caged compounds can be used to produce rapid and uniform changes in the cytosolic concentration of photolysis products (reviewed in McCray and Trentham, 1989; Homsher and Millar, 1990; Kaplan, 1990; Somlyo and Somlyo, 1990). We have used flash photolysis to produce concentration jumps of ATP and Ins(1,4,5)P_3 in *Limulus* ventral photoreceptors. A large portion of our paper is devoted to methods used to uncouple the phototransduction cascade at initial steps, as a preliminary to flash photolysis experiments. These procedures are necessitated by the massive excitation normally produced by the near ultraviolet flash used for photolysis. Our results suggest that photoreleased IP_3, but not photoreleased ATP, releases calcium ions from intracellular stores and the resulting increase in $[Ca^{2+}]_i$ enhances the amplification of the phototransduction cascade.

Some of the results presented in this paper have appeared previously in abstract form (Faddis and Brown, 1992).

METHODS

Electrical Recording

The methods used for dissection and voltage clamp of single photoreceptors from the ventral rudimentary eye of *Limulus polyphemus* were essentially the same as those used by Millecchia and Mauro (1969*a*, *b*). Briefly, single ventral eyes were dissected and pinned with glass fibers to Sylgard 184 (Dow Corning Corp., Midland, MI) in a perfusion chamber. Photoreceptors used

for these experiments were positioned to protrude from a side of the nerve to optimize stimulation and viewing with an inverted microscope. The preparation was transilluminated with infrared light (λ = 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, Tokyo, Japan) and displayed on a video monitor to make visible intracellular injections and microelectrode impalements. Microelectrodes were pulled from borosilicate glass (1.2 mm o.d., 1.1 mm i.d.) and typically had a resistance of 5–8 MΩ when filled with 2 M KCl. To prepare cells for microelectrode impalements, the preparation was briefly (60 s) bathed in 2% Pronase (grade B; Calbiochem Corp., La Jolla, CA) in artificial sea water (ASW) at room temperature (~22°C) and then washed 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. During experiments, the preparation was continuously superfused with ASW at a rate of 2 ml/min (bath volume = 1.5 ml). Experiments were performed at room temperature except as otherwise noted. Temperature jumps to 5°C were made by cooling the superfusate with a thermoelectrically cooled manifold. The temperature of the superfusate was monitored continuously by a thermocouple placed next to the biological preparation. Cells were voltage clamped by the two-electrode technique (Millecchia and Mauro, 1969*b*; Brown and Coles, 1979; Brown, Harary, and Waggoner, 1979). In experiments in which photoreceptors were exposed to hydroxylamine, 200 mM NaCl was replaced with 200 mM hydroxylamine hydrochloride; the pH of the solution was then adjusted to 6.5 with solid NaOH.

Intracellular Injection

Intracellular injections were made by applying brief (50 ms) pressure pulses (10–30 lb/in^2) to the back of microelectrodes back-filled with ~2 μl of injection solution. A successful injection produced an optical disturbance in the cytoplasm of the cell clearly visible on the video monitor (Corson and Fein, 1983*b*). Injection pressure was adjusted to produce the smallest injection that could be reliably seen. The average volume per injection (using the visual criterion described above) was measured to be 1.3 ± 0.31 pl (±SD, n = 5 cells) with a radioactive isotope of known specific activity as described previously (Brown and Blinks, 1974). The average injection size used in the experiments described in this paper was ~1% of the average intracellular volume estimated from ultrastructural studies (Clark et al., 1969; Calman and Chamberlain, 1982).

All compounds except BAPTA ([1,2-bis-[*o*-amino-phenoxy]ethane-*N*-*N*-*N'*-*N'*] tetraacetic acid) were dissolved in a standard injection buffer made of (mM) 300 potassium acetate and 10 MOPS (morpholinopropane sulfonic acid), pH 7.3. Compounds injected were: GDPβS (guanosine 5'-*O*-[2-thiodiphosphate]) and heparin (sodium salt, grade II, from porcine intestinal mucosa, #H7005) from Sigma Chemical Co. (St. Louis, MO); *myo*-inositol 1,4,5-trisphosphate, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester (caged Ins[1,4,5]P_3), and adenosine-5'-triphosphate, and P^3-1-[2-nitrophenyl]ethyl ester (caged ATP) from Calbiochem Corp.; and BAPTA (potassium salt) from Molecular Probes, Inc. (Eugene, OR). Due to the considerable lability of GDPβS, this compound was ordered shipped on dry ice in the minimum quantity per vial (1 mg) and stored desiccated at −80°C. For each experimental day it was to be used, a new vial of GDPβS was opened, dissolved in injection buffer, and refrozen (−20°C). This solution was thawed to fill microelectrodes during a single experiment. BAPTA injection solution contained (mM) 300 BAPTA (tetrapotassium salt) and 10 MOPS, pH 7.3.

Flash Photolysis

The optical setup used for flash photolysis experiments is shown schematically in Fig. 1. The output of the xenon flashlamp was collimated with a large aperture quartz lens, filtered to exclude damaging short wavelength ultraviolet light (excluded λ < 300 nm) and excess actinic

light (excluded $\lambda > 400$ nm), combined with the infrared illumination ($\lambda = 950$ nm) used to view the preparation, and focused onto the preparation with a quartz condenser lens. With this setup, a full-power flash delivered ~30 mJ per flash to the focus of the quartz condenser lens (estimate obtained by integrating trace in Fig. 1 *B*). The intensity of the flashlamp was related to that of the tungsten–halogen source as described previously (Brown and Coles, 1979).

An estimate of the concentration jump of ATP per full-power flash produced inside single photoreceptors from injected caged ATP was made as follows. A small volume (1 μl) of 10 mM caged ATP solution in injection buffer was put into a quartz capillary (1.2 mm o.d., 1.0 mm i.d.) and placed in the flash photolysis setup with the bottom of the fluid column in the plane of

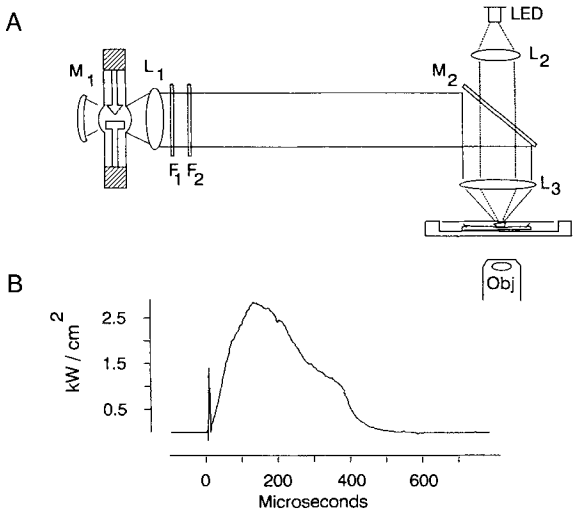

FIGURE 1. (*A*) Setup for flash photolysis experiments. Relevant optical elements are: xenon flashlamp (model 35S lamp, Strobex model 238 power supply; Chadwick-Helmuth, El Monte, CA); M_1, spherical mirror in lamp housing; L_1, quartz lens (75 mm in diameter, $f = 40$ mm); F_1, WG295 filter, band pass >300 nm (Schott Optical Glass, Duryea, PA); F_2, Schott UG11 filter, band pass between 250 and 400 nm; M_2, dichroic beamsplitter, for 45° incidence >500 nm = transmission, <400 nm = reflection) (Omega Optical Inc., Brattleboro, VT); L_2, 15-mm glass lens ($f = 50$ mm); L_3, 35-mm quartz lens ($f = 20$ mm); LED, infrared light–emitting diode ($\lambda = 950$ nm, type GAL 10; Plessey Solid State, Irvine, CA) used for transilluminating the biological preparation; and Obj, microscope objective (Zeiss LD-Epiplan 16×/n.a. = 0.33) on the inverted microscope (model IM35; Carl Zeiss, Inc., Thornwood, NY). A CCD TV camera (not shown) was used to view the IR image formed by the microscope objective. The output of a tungsten–halogen light source (not shown) used to produce dim flash stimuli was focused on the preparation through the microscope objective via the epifluorescence optics of the microscope. (*B*) The time course of flash energy was measured in the focal plane of the biological preparation with a fast photodiode (MRD 510; Motorola, Phoenix, AZ). The focus of the flashlamp approximately filled the active area of the photodiode.

focus normally occupied by an injected photoreceptor. After a photolysis flash, free ATP was separated from caged ATP by HPLC (Walker, Feeney, and Trentham, 1989) on a Partisil 10 SAX column eluted isocratically with 0.3 M ammonium phosphate, pH 5.4, and methanol (19:1, vol/vol); the absorbance ($\lambda = 254$ nm) of the column eluate was monitored continuously. The integrated area of each peak together with the appropriate molar absorption coefficients (for ATP $\epsilon = 15{,}400\ M \cdot cm^{-1}$; for caged ATP $\epsilon = 18{,}900\ M \cdot cm^{-1}$) was used to calculate that a concentration jump of ~400 μM ATP/flash was produced (in each of two experiments). In parallel, a sample kept in the dark at room temperature for 3 h did not undergo measurable hydrolysis. We expect that a photolysis flash would produce a comparable concentration jump

per flash for caged Ins[1,4,5]P_3 due to its similar photochemistry (Walker et al., 1989). Within a photoreceptor, the concentration of caged compound was probably near 1 mM after several intracellular injections. Therefore, the intracellular concentration jump due to photolysis of caged compound is estimated to be ~40 μM. This estimate might be low because self-absorption of the caged compounds is reduced inside a cell (compared with the inside of the 1-mm-diam capillary) by both the reduced intracellular concentration and the shorter path length.

Stimulation

Stimulus light was provided 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) when attenuated 7.8 log units with neutral density filters. The unattenuated stimulus beam energy was 1.4 mW/cm^2 measured in the specimen plane. In some experiments, a high pressure xenon flash lamp was used to evoke early receptor potentials and to produce saturating stimuli for voltage clamped photoreceptors.

RESULTS

Ultraviolet Flash Used for Photolysis Saturates the Photocurrent

Initially, experiments were performed to determine the extent to which ventral photoreceptors were stimulated by the light used for flash photolysis of caged compounds (band pass 300–400 nm). Isolated photoreceptors that had been dark-adapted a minimum of 30 min were voltage clamped to their dark resting potential. A typical voltage clamp current activated by a flashlamp discharge is shown in Fig. 2*A*. The amplitude of the average response was 1121.9 ± 252.5 nA (mean ± SD for n = 12 cells). The voltage clamp current began after the flash with a latency of 10.3 ± 2.56 ms (mean ± SD for n = 12 cells) comparable to previously reported latencies for flashes exciting all available rhodopsin molecules in a cell (Brown and Coles, 1979). In a separate experiment, the proportion of the total rhodopsin molecules activated in a typical cell by the photolysis flash was estimated by measuring the amplitude of the negative component of the early receptor potential (ERP). In two cells, the ERP amplitude was ~90% of its predicted value at saturation; thus, ~90% of the available rhodopsin (equal to 50% of the rhodopsin in a cell; Lisman and Sheline, 1976) was converted to metarhodopsin by the flash.

Because the intensity of the photolysis flash is sufficient to activate almost all rhodopsin in a photoreceptor, many subsequent steps in the phototransduction cascade may be near saturation. In this condition, small concentration jumps derived from flash photolysis of caged compounds are likely to be ineffective. In addition, the latencies of the photocurrents elicited by bright flashes are similar to estimates for the time constants of product liberation for the caged compounds used in this study: 4 ms for caged Ins(1,4,5)P_3 (Walker et al., 1989) and 9 ms for caged ATP (calculated for pH 7.3 from McCray, Herbette, Kihara, and Trentham, 1980). Therefore, we attempted to develop a procedure which would uncouple phototransduction at early steps in the cascade but leave later steps intact.

Rhodopsin Bleaching with Hydroxylamine

We reasoned that the most desirable prelude to experiments with caged compounds would be to inactivate, as completely as possible, the initial step in the phototransduction cascade, i.e., the photoactivation of rhodopsin. In isolated vertebrate photoreceptors, this problem is trivial; the photoproduct of rhodopsin activation (metarhodopsin) is thermally unstable at room temperature and hydrolyzes to opsin and free all-*trans* retinal (reviewed in Morton, 1972). The resultant desensitization is irreversible in the absence of exogenously added 11-*cis*-retinal (Jones, Crouch,

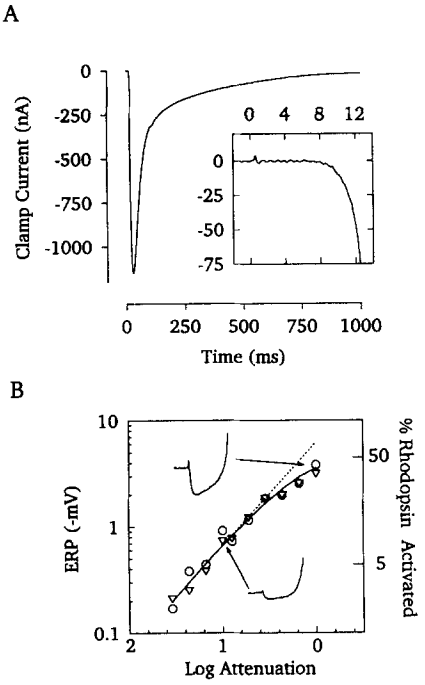

FIGURE 2. Responses of dark-adapted ventral photoreceptors to the light flash used for photolysis. (*A*) Voltage clamp current induced by discharge of the flashlamp. The cell was voltage clamped to its dark membrane potential (−50 mV). The output of the flashlamp was filtered to a band pass of 300–400 nm. The inset is an enlargement of the record to illustrate the latency of the response. (*B*) Log-log plot of the maximum amplitude of the negative phase of the early receptor potential as a function of the flash attenuation for two consecutive experimental runs through the sequence of flash attenuations (circles and triangles) for a different cell than *A*. The solid line is the plot of the equation (fit by eye to the data) describing the relation between stimulus intensity (I) and ERP amplitude: $ERP = ERP_{sat} \cdot (1 - e^{-\beta I})$, where β is a constant proportional to the photosensitivity of the rhodopsin molecules and ERP_{sat} is the maximum amplitude corresponding to the conversion of 50% of the rhodopsin molecules in the cell (derived from Lisman and Bering, 1977, Eq. 1). The dashed line in the figure is a straight line with unity slope fit by eye to the responses to the four lowest intensity flashes. The insets show the ERP responses versus time (25 ms total duration) for the two points indicated by the arrows.

Wiggert, Cornwall, and Chader, 1989). In contrast, extensive illumination of *Limulus* ventral eye produces a photoequilibrium of rhodopsin and thermally stable metarhodopsin with little concomitant change in the sensitivity to light (Lisman and Sheline, 1976; Lisman and Strong, 1979). Therefore, we sought to develop a chemical protocol for inactivating rhodopsin.

Certain aromatic amines have been used to block rhodopsin regeneration in vertebrate retina; *p-n*-butylaniline is one of the most active of these compounds (Bernstein, Fulton, and Rando, 1986*a*; Bernstein, Lichtman, and Rando, 1986*b*).

However, in *Limulus* ventral eye, a 30-min incubation in 1 mM *p-n*-butylaniline together with illumination (1 $\mu W/cm^2$) had no effect on the sensitivity to light ($n = 2$ cells) measured after a subsequent 30 min of dark adaptation. The lack of an effect of *p-n*-butylaniline on the photosensitivity in *Limulus* photoreceptors is probably related to the development of a photoequilibrium between rhodopsin and metarhodopsin during prolonged illumination and the maintenance of that photoequilibrium during subsequent dark adaptation (Lisman and Sheline, 1976). In vertebrate retina, in contrast to *Limulus* ventral eye, dark adaptation after bright illumination requires rhodopsin regeneration from opsin and free 11-*cis*-retinal; *p-n*-butylaniline and related compounds block this process by catalyzing the formation of all-*trans* retinal from free 11-*cis*-retinal (Bernstein et al., 1986*a*).

We attempted to use hydroxylamine as a reagent to react with and remove the chromophore from rhodopsin molecules in intact photoreceptors from *Limulus*. Hydroxylamine has been used to obtain a rhodopsin photobleaching difference spectrum from a *Limulus* lateral eye extract (Hubbard and Wald, 1960). Hydroxylamine has also been used to bleach rhodopsin to completion in squid rhabdomeric membranes illuminated with bright light (Seki, Hara, and Hara, 1980). To monitor the extent of rhodopsin inactivation, we used both the early receptor potential and the quantum efficiency of bump production (assayed as the proportionality constant relating bump frequency and light intensity) as indices of rhodopsin content. Initially, a procedure similar to that described by Seki et al. (1980) was used. Single ventral nerves were dark-adapted for at least 30 min. A number of identified, isolated cells along the length of the nerve were impaled with a single microelectrode. Both the responses to flashes of progressively increasing intensities and the early receptor potential in response to the unattenuated discharge of the flashlamp were recorded from each cell. The ventral nerve was then cooled to 5°C, superfused with 200 mM hydroxylamine hydrochloride in ASW, and illuminated with intense white light (~ 30 mW/cm^2) for 60 min. In later experiments, we found that a 10-min exposure to hydroxylamine was as effective and minimized toxic effects of prolonged exposure. The preparation was then returned to darkness, superfused with ASW, and warmed to room temperature. After a minimum of 45 min of dark adaptation, cells were identified using a map of their locations along the ventral nerve, the cells were reimpaled with a microelectrode, and the responses to flashes were again recorded. This procedure completely abolished the early receptor potential measured in 20 cells and reduced the quantum efficiency 325 ± 90-fold (mean ± SD) for $n = 12$ cells. Fig. 3 is an example of the range of the change in quantum efficiency observed in a single ventral nerve. The change in quantum efficiency observed in this ventral nerve ranged from 80- to 180,000-fold (median = 110-fold for $n = 7$ cells). The range in the response to hydroxylamine bleaching may reflect differing amounts of rhodopsin in internal rhabdomes (Calman and Chamberlain, 1982); rhodopsin in internal rhabdomes may not be as accessible to superfused hydroxylamine as rhodopsin in external rhabdomes.

In contrast to the response to hydroxylamine superfusion together with intense illumination, superfusion of hydroxylamine in darkness was entirely ineffective. Fig. 4 is an example of responses to dim lights and early receptor potentials recorded from three cells on a single ventral nerve (the other of the pair of nerves from the same

animal used for Fig. 3) before and after hydroxylamine exposure in darkness. The smaller amplitudes of both the responses to dim light and the ERPs after the cells were treated with hydroxylamine reflect a smaller input resistance that was probably caused by multiple microelectrode impalements. The results presented in Figs. 3 and 4 suggest that *Limulus* rhodopsin, like squid rhodopsin (Hubbard and St. George, 1958), is resistant to attack by hydroxylamine in the dark. Presumably, light induces a conformational change in rhodopsin that enables the attack by hydroxylamine on the retinal.

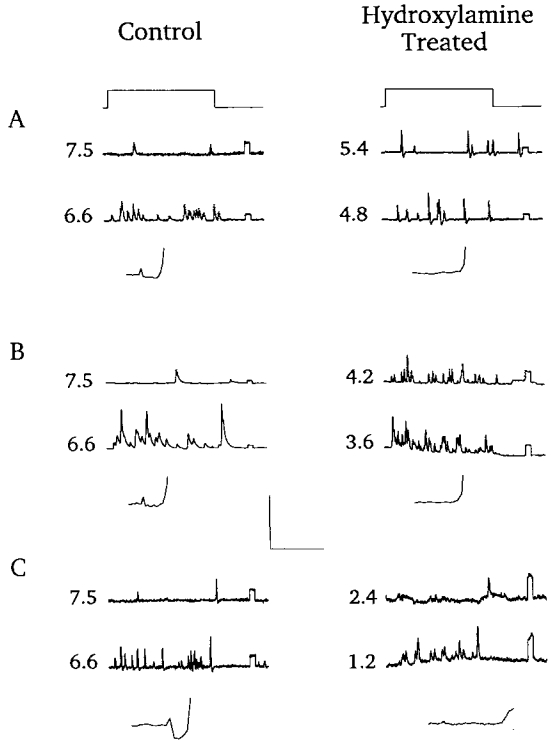

FIGURE 3. Hydroxylamine in the presence of bright light reduces the quantum efficiency of phototransduction. Responses elicited by dim flashes and ERPs recorded from three cells (labeled *A*, *B*, and *C*) before and after exposure to 200 mM hydroxylamine (at 5°C for 10 min) with bright illumination. The three cells (from a set of seven cells) labeled *A*, *B*, and *C*, were from a single ventral nerve and represent, (respectively), the least, median, and greatest effect of the treatment. The record at the top of each column shows a monitor of the stimulus flashes (5 s duration). The top two records of membrane voltage in each set show discrete events whose average rate is approximately proportional to the photon flux incident on the photoreceptor. Light intensities are given to the left of each record expressed as the log attenuation of the tungsten source. The calibration pulse at the end of each record is 2 mV, 300 ms. The bottom record in each set shows the ERP (elicited by an unattenuated flashlamp discharge). Calibration bars for ERPs only: 10 mV and 25 ms. The large positive depolarization at the end of the ERP record marks the beginning of the late receptor potential.

We also determined the effect of bleaching with hydroxylamine on the stimulus–response relation. Dark-adapted photoreceptors were voltage clamped to their dark membrane potential. Responses were recorded to a series of progressively brighter light flashes from the tungsten source (50 ms duration) and unattenuated flashes from the xenon flashlamp. The electrodes were then withdrawn and the cells were exposed to hydroxylamine and intense steady illumination. After 45 min of dark

adaptation, cells were reimpaled with two microelectrodes and voltage clamped to the same membrane potential as in the control condition, and the responses to light flashes were again recorded. Fig. 5 shows records from a typical cell in which bleaching with hydroxylamine shifted the stimulus–response relation 2.6 log units. The range of the shift for all cells tested was 2.1–3.2 log units (for $n = 3$ cells). These shifts are comparable in magnitude to the change in quantum efficiency described above. The waveforms of the responses recorded after hydoxylamine treatment were approximately the same as those of responses of comparable amplitude before

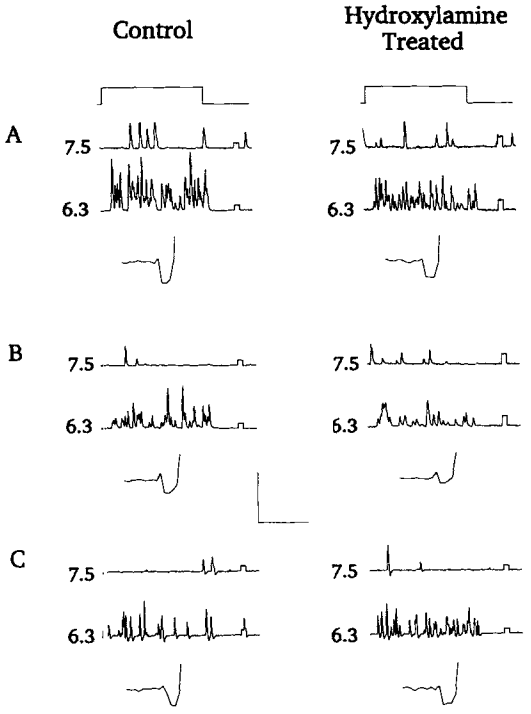

FIGURE 4. Hydroxylamine in darkness does not reduce the quantum efficiency of phototransduction. Responses elicited by dim flashes and ERPs recorded from three cells (labeled *A*, *B*, and *C*) before and after exposure to 200 mM hydroxylamine (at 5°C for 10 min) in darkness. The three cells (from a set of seven cells) labeled *A*, *B*, and *C*, were from a single ventral nerve (the other of the pair from the same animal as that used for the recordings shown in Fig. 3). The record at the top of each column shows a monitor of the stimulus flashes (5 s duration). The top two records of membrane voltage in each set show discrete events whose average rate is approximately proportional to the photon flux incident on the photoreceptor. Light intensities are given to the left of each record expressed as the log attenuation of the tungsten source. The calibration pulse at the end of each record is 2 mV, 300 ms. The bottom record in each set shows the ERP (elicited by an unattenuated flashlamp discharge). Calibration bars for ERPs only: 10 mV and 25 ms.

treatment (Fig. 5 *B*). Although photoreceptors were desensitized to dim stimuli by several log units, the photocurrents induced by unattenuated flashlamp discharges were 98.1 ± 2.2% (mean ± SD for $n = 3$ cells) of the control values. As a result, further experiments were performed to identify an adjunct to bleaching with hydroxylamine in order to uncouple phototransduction more completely.

Photoreceptor Desensitization by GDPβS

G protein activation has been proposed to link rhodopsin excitation to later steps in the phototransduction cascade of *Limulus* ventral eye (Fein and Corson, 1979, 1981;

Bolsover and Brown, 1982; Corson and Fein, 1983*a* ; Corson, Fein, and Walthall, 1983). We attempted to uncouple phototransduction by disabling G protein function with GDPβS. GDPβS is a metabolically stable analogue of GDP that has been used to block G protein activation (Eckstein, Cassel, Levkovitz, Lowe, and Selinger, 1979), probably through stabilization of the inactive, GDP-bound form of the G protein complex (Van Dop, Yamanata, Steinberg, Sekura, Manclark, Stryer, and Bourne,

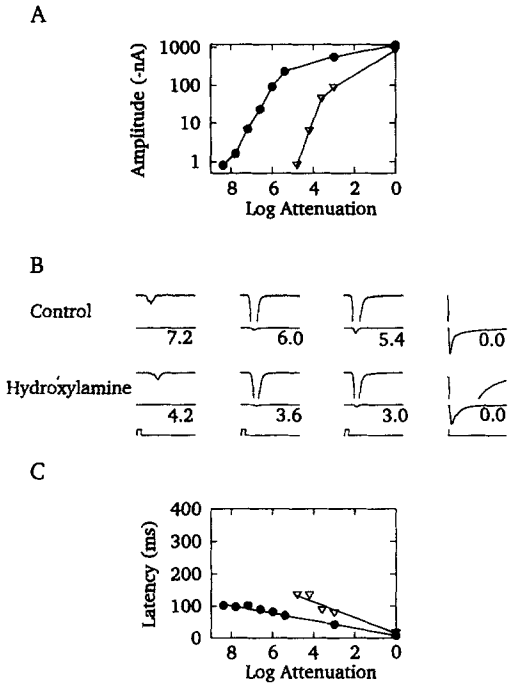

FIGURE 5. Effect of hydroxylamine on the stimulus–response relation for brief flashes in a voltage clamped photoreceptor. A single cell was voltage clamped to its dark resting potential (−48 mV). The log attenuation of 50 ms flashes (from the tungsten–halogen source) is expressed relative to the unattenuated xenon flashlamp (0.0) as described in Methods. An interflash interval of 60 s was used for dim flashes and 120 s for bright flashes. (*A*) The peak amplitude of the voltage clamp current induced by each flash is plotted as a function of the log attenuation before (●) and after (▽) bleaching with hydroxylamine. (*B*) Representative records of voltage clamp current across the range of stimulus attenuations plotted in *A* are shown before and after bleaching with hydroxylamine. Each trace is plotted at two gains differing by a factor of 50. The bottom record of each set is a stimulus monitor; the amplitude of each stimulus pulse is equivalent to 5 nA (high gain traces) or 250 nA (low gain traces) with a duration of 50 ms. The log attenuation of the stimulus flash is given below the record. (*C*) Latency of the voltage clamp currents plotted as a function of stimulus intensity before (●) and after (▽) bleaching with hydroxylamine. The latency for each response plotted in *A* was measured as the time interval between light onset and the first detectable change in voltage clamp current. A linear regression is also plotted (solid lines through data) for each condition.

1984). Intracellular injection of GDPβS has been demonstrated to desensitize *Limulus* ventral photoreceptors by several log units (Bolsover and Brown, 1982; Fein, 1986; Kirkwood, Weiner, and Lisman, 1989). However, previous studies in *Limulus* ventral eye have not examined either the effect of GDPβS injection on the response to bright light or whether it is feasible to titrate all available G proteins by injection of GDPβS to a high intracellular concentration.

To address these issues, the effects of injection of GDPβS to high intracellular concentration were assayed in *Limulus* ventral photoreceptors. Fig. 6 shows examples of receptor potentials recorded in response to dim flashes before and after multiple injections of GDPβS (20 injections for the cell in *A*, 17 injections for the cell in *B*). A striking characteristic of the effect of GDPβS is the steepness of the stimulus–response relation to the dimmest effective stimuli. In 9 of 12 cells injected, responses to the dimmest effective stimuli typically appeared to be clusters of very large

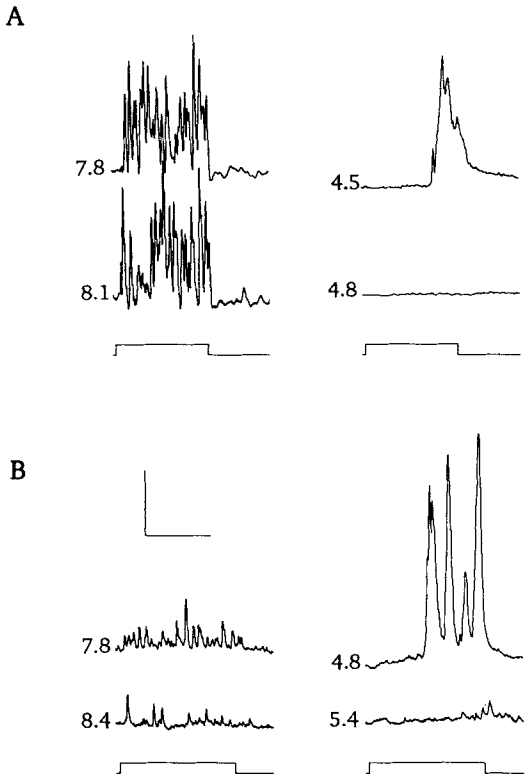

FIGURE 6. Receptor potentials evoked by dim light flashes before (*left side*) and after (*right side*) GDPβS injection in two cells, *A* and *B*. Photoreceptors were dark-adapted at least 30 min before impalement with a single microelectrode filled with 2 M KCl. The responses to dim flashes (3 s duration, 1 every 30 s) were recorded and the electrode was withdrawn. The cell was then reimpaled with another microelectrode that contained 25 mM GDPβS in injection buffer and 10 injections were made. The responses to dim flashes were again recorded; the dimmest flashes that evoked responses were shifted by several log units to higher intensity (records on the right). The log attenuation of the stimulus for each record is given relative to the unattenuated tungsten source. The vertical scale bar is equal to 6 mV in *A* and 4 mV in *B*. The horizontal scale bar is equal to 200 ms in *A* and *B*. The bottom trace in each set is a stimulus monitor.

quantum bumps. These clusters of large quantum bumps occurred after a long latency from light onset (often > 1 s) and were followed by desensitization of the cell for several minutes. In contrast to the response to dim lights, injected cells responded to bright flashes with qualitatively normal receptor potentials as shown in Fig. 7.

In uninjected *Limulus* photoreceptors the rate of quantal events is approximately linearly related to light intensity (Fuortes and Yeandle, 1964). Deviations from this linear relationship are relatively small over several orders of magnitude of stimulus

intensity (Dodge, Knight, and Toyoda, 1968; Srebro and Behbehani, 1972). These findings are consistent with the proposal that each excited rhodopsin molecule evokes a single discrete event (Fuortes and Yeandle, 1964) and that the quantum efficiency (defined as the number of discrete events evoked per photon absorbed) is constant at all light intensities. A possible explanation for the steepness of the stimulus–response relation in cells injected with high concentrations of GDPβS is that quantum efficiency becomes a highly cooperative function of light intensity.

To test this hypothesis, the relation between quantum bump frequency and stimulus intensity (20 s duration flashes separated by 2 min) was measured in dark-adapted photoreceptors before and after intracellular injection of GDPβS. For five cells tested, the data were reasonably well fit by a straight line both before and after injection (the regression coefficients were: control $r^2 = 0.925 \pm 0.023$, GDPβS injected $r^2 = 0.872 \pm 0.051$, mean values ± SEM). For the same five cells, the

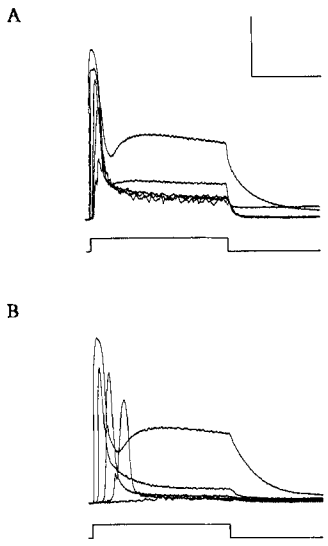

FIGURE 7. Receptor potentials recorded in a single cell to bright flashes before (*A*) and after (*B*) 20 injections of GDPβS. *A* and *B* each contain a set of five records of receptor potentials in response to a series of flash intensities (1 every 30 s, 3 s duration, log attenuation of the stimulus = 5.4, 4.8, 4.2, 3.0, 1.2). The scale bars are equal to 20 mV and 1,500 ms. The bottom trace in each set is a stimulus monitor.

changes in quantum efficiencies were determined (as the ratio of the slopes of the lines before and after injection). The range of the reduction in quantum efficiency was 10–3,820-fold (mean value = 1,064-fold; $n = 5$ cells), similar to that previously reported (Kirkwood et al., 1989). These data do not support the hypothesis that the cause of the change in shape of the stimulus–response relation after injection of GDPβS is a dependence of quantum efficiency on light intensity. The data suggest that the quantum efficiency after injection of GDPβS is still approximately independent of light intensity, although the magnitude of the quantum efficiency is reduced.

Ventral photoreceptors contain a full complement of voltage-activated conductances in addition to the light-activated conductance (reviewed in Fain and Lisman, 1981). The steepness of the stimulus–response relation in photoreceptors injected with GDPβS might be attributable to an effect of GDPβS on voltage-dependent conductances that modulate the waveform of the receptor potential. To test this idea,

voltage clamp current was measured before and after intracellular injection of GDPβS. Fig. 8 *A* is a log-log plot of the peak voltage clamp current recorded in a single cell in response to brief flashes before and after 10 small injections of GDPβS. The intensity of each flash is plotted in units relative to a full-power flashlamp discharge. The plot demonstrates that the slope of the stimulus–response relation for

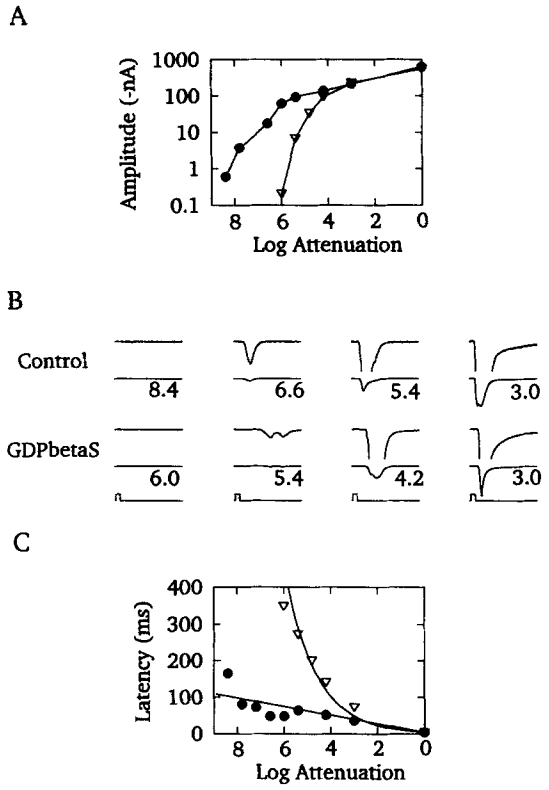

FIGURE 8. Effect of GDPβS on the stimulus–response relation for brief flashes in a voltage clamped photoreceptor. A single cell was voltage clamped to its dark resting potential (−55 mV). The log attenuation of 50-ms flashes (from the tungsten–halogen source) is expressed relative to the unattenuated xenon flashlamp (0.0) as described in Methods. An interflash interval of 60 s was used for dim flashes and 120 s for bright flashes. (*A*) The peak amplitude of the voltage clamp current induced by each flash is plotted as a function of the log attenuation before (●) and after (▽) 10 injections of 25 mM GDPβS in injection buffer. (*B*) Representative records of voltage clamp current across the range of stimulus attenuations plotted in *A* are shown before and after injection of GDPβS. Each trace is plotted at two gains differing by a factor of 10. The bottom record of each set is a stimulus monitor; the amplitude of each stimulus pulse is equivalent to 5 nA (high gain traces) or 50 nA (low gain traces) with a duration of 50 ms. The log attenuation of the stimulus flash is given below each record. (*C*) Latency of the voltage clamp currents plotted as a function of stimulus intensity before (●) and after (▽) injection of GDPβS. The latency for each response plotted in *A* was measured as the time interval between light onset and the first detectable change in voltage clamp current. A linear regression (for control) or a single exponential (for GDPβS injected) is plotted as a solid line for each condition.

relatively dim stimuli is steeper after intracellular injection of GDPβS. A similar increase in the slope of the stimulus–response relation was observed in three additional cells out of a total of six cells studied. In the two cells that did not exhibit a change in the shape of the stimulus–response relation, the stimulus–response relation for relatively dim stimuli was shifted the least (1.20 and 1.26 log units). In

the four cells that exhibited a change in the shape of the stimulus–response relation, the shift for relatively dim stimuli ranged from 1.38 to 3.65 log units (mean = 1.91 ± 0.76 log units, mean ± SD). In two of these latter cells, the shift in the stimulus–response relation was measured after each injection of GDPβS. In both cells, the shift was largely accomplished by the first two injections, with little effect by additional injections. At brighter intensities, the current amplitudes after intracellular injection of GDPβS quickly approach the control values. The response to an unattenuated flash after intracellular injection of GDPβS was 76.4 ± 11.8% (mean ± SD for $n = 6$ cells) of the control value. These voltage clamp data suggest that the steepness of the stimulus–response relation apparent in current clamp records (see Fig. 7) is not related to an effect of the GDPβS on voltage-activated conductances but is due to a direct effect on the transduction cascade.

In addition to desensitization, there is a striking effect of GDPβS on the latency of the photocurrent induced by the dimmest effective stimuli. Before GDPβS injection, a plot of photocurrent latencies vs. log stimulus attenuation is well fit by a linear function (Fig. 8 *C*, circles). However, after GDPβS injection, a plot of photocurrent latencies vs. log stimulus attenuation is better approximated by a single exponential function (Fig. 8 *C*, triangles). Photoreceptors that had been desensitized with hydroxylamine to the same extent as photoreceptors injected with GDPβS did not exhibit a similar effect on the photocurrent latency (compare Fig. 5 *C* with Figs. 8 *C* and 10 *C*).

Photoreceptor Desensitization by GDPβS and Hydroxylamine Is Additive

Although both injecting a cell with GDPβS and bleaching it with hydroxylamine produced large changes in quantum efficiency, the photocurrent induced by the unattenuated flashlamp discharge after either treatment alone was nearly unchanged in amplitude and latency. Therefore, we attempted to determine whether the two treatments could be used together on a single cell to produce a greater degree of desensitization. Dark-adapted photoreceptors were impaled with a single microelectrode, and the responses to a series of progressively brighter flashes were recorded. Photoreceptors were then bleached by treatment with hydroxylamine. After dark adaptation, the responses to progressively brighter light flashes were recorded both before and after several injections of GDPβS. Fig. 9 shows receptor potentials recorded from a single cell in response to a range of light intensities before and after each of the treatments. The quantum efficiency of this cell was reduced ~1,000,000-fold after bleaching with hydroxylamine and intracellular injection of GDPβS; however, the waveforms of the responses were largely normal. After GDPβS injection, this cell exhibited a marked compression of the dynamic range of its responses relative to both the control condition and after hydroxylamine bleaching; this characteristic was frequently observed in cells treated by GDPβS injection alone (as described above). The quantum efficiency for cells treated by the combined protocol was reduced on average 870,000 ± 660,000-fold (mean ± SD for $n = 10$ cells).

The shift in the stimulus–response relation of the light-activated current was measured before and after hydroxylamine treatment combined with GDPβS injection. Fig. 10 *A* is a log-log plot of the peak voltage clamp current recorded in a single cell in response to brief flashes before and after bleaching with hydroxylamine and injection of GDPβS. The intensity of each flash is plotted in units relative to a

flashlamp discharge (at full power). The slope of the stimulus–response relation for relatively dim stimuli after treatment with hydroxylamine and GDPβS is steep relative to control; this characteristic was also observed in cells treated with GDPβS alone (see Fig. 8*A*). For this cell, the stimulus–response relation was shifted by ~4.2 log units (measured in the middle of the linear range of the relation) after treatment. The mean shift in the stimulus–response relation was 4.4 ± 0.9 log units (mean ± SD for $n = 6$ cells, maximum response = 6.0 log units). This value is approximately equal to the sum of the mean shifts obtained for hydroxylamine bleaching alone and GDPβS injection alone. The amplitude of the photocurrent in response to an unattenuated

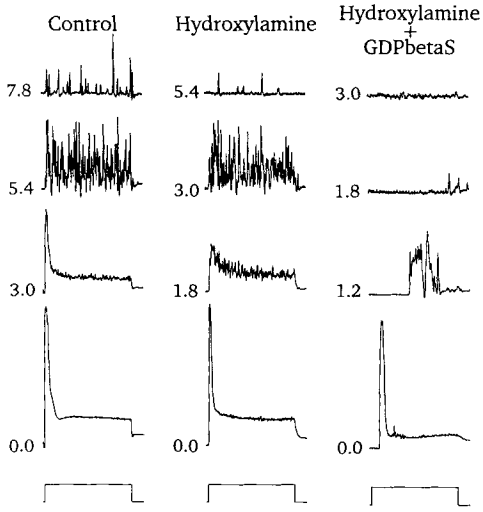

FIGURE 9. Receptor potentials recorded in a single cell in three conditions: control, after bleaching with hydroxylamine and light, and after both hydroxylamine bleaching and intracellular injection of GDPβS. The cell was dark-adapted for 30 min before impalement with a single microelectrode. Receptor potentials were recorded in response to flashes of progressively increasing intensity (1 every 30 s, 5 s duration), spanning the range of the stimulus–response relation; selected records are displayed in the column labeled Control with the attenuation of the stimulus (expressed relative to the unattenuated tungsten source) given to the left of each record. The electrode was withdrawn and the cell was bleached with 200 mM hydroxylamine and bright light (at 5°C for 10 min). After dark adaptation (~45 min) the cell was reimpaled with a microelectrode containing 25 mM GDPβS in injection buffer. Receptor potentials in response to flashes of progressively increasing intensities were recorded; selected records are displayed in the column labeled Hydroxylamine. The cell was then dark-adapted and injected with 10 injections of GDPβS solution, and receptor potentials were recorded again; selected records are displayed in the column labeled Hydroxylamine + GDPbetaS. The bottom record in each column is a stimulus monitor. The amplitude of the stimulus monitor record is equivalent to 2 mV for the top two rows of records and 6 mV for the third and fourth rows of records.

flashlamp discharge was reduced to 28.3 ± 29.6% (mean ± SD for $n = 5$ cells) of the control value after treatment with hydroxylamine and GDPβS injection. The photocurrent latency after flashlamp discharge was 8.4 ± 1.4 ms before treatment and 26.0 ± 8.7 ms after treatment (for the same set of five cells).

Flash Photolysis of Caged Compounds in Photoreceptors in Which Phototransduction Is Largely Uncoupled

Injection of Ins[1,4,5]P_3 into the rhabdomeric lobe ventral photoreceptors induces the opening of a conductance with properties similar to those of the light-induced

conductance (Brown et al., 1984; Fein et al., 1984). The injection of Ins[1,4,5]P_3 is largely ineffective after prior intracellular injection of a calcium chelator (EGTA and BAPTA: Rubin and Brown, 1985; Quin 2 and EGTA: Payne, Corson, Fein, and Berridge, 1986*b*); this finding suggests that Ins[1,4,5]P_3 acts indirectly on ion channels in the plasma membrane through an increase in intracellular free calcium.

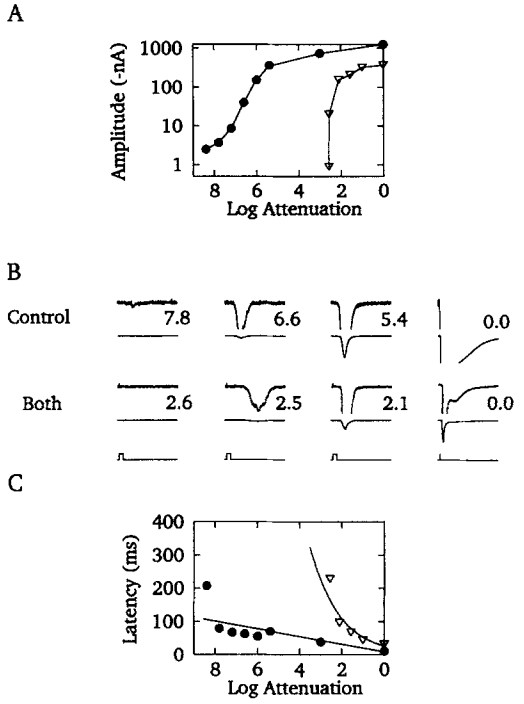

FIGURE 10. Effect of GDPβS in combination with hydroxylamine on the stimulus–response relation. All parts of the figure were derived from measurements made from a single cell voltage clamped to its dark resting potential (−61 mV). The log attenuation of 50 ms flashes (from the tungsten–halogen source) is expressed relative to the unattenuated xenon flashlamp (0.0) as described in Methods. An interflash interval of 60 s was used for dim flashes and 120 s for bright flashes. (*A*) The peak amplitude of the voltage clamp current induced by each flash is plotted as a function of the log attenuation before (●) and after (▽) hydroxylamine treatment plus 10 injections of 25 mM GDPβS in injection buffer. (*B*) Representative records of voltage clamp current across the range of stimulus attenuations plotted in *A* are shown before and after hydroxylamine treatment plus injection of GDPβS. Each trace is plotted at two gains (differing by a factor of 20). The bottom record of each set is a stimulus monitor; the amplitude of each stimulus pulse is equivalent to 5 nA (high gain traces) or 50 nA (low gain traces) with a duration of 50 ms. The log attenuation of the stimulus flash is given below each record. (*C*) Latency of the voltage clamp currents plotted as a function of stimulus intensity before (●) and after (▽) the combined treatments of hydroxylamine and injection of GDPβS. The latency for each flash plotted in *A* was measured as the time interval between light onset and the first detectable change in voltage clamp current. A linear regression (for control) or a single exponential (for the combined treatments) is plotted as a solid line for each condition.

However, an alternative suggestion is that Ins[1,4,5]P_3 both directly gates ion channels in the plasma membrane of ventral photoreceptors and causes calcium release by gating channels in the membranes of intracellular organelles. For injected Ins[1,4,5]P_3, the latter process may produce the dominant effect. Flash photolysis of caged Ins[1,4,5]P_3 might help to distinguish between these two suggestions on the

basis of the latency of the response. If Ins[1,4,5]P_3 directly gates a channel in the plasma membrane, an inward current would be expected to activate with little or no latency after the flash. By analogy, photolysis of caged cGMP opens channels in membrane patches from rod outer segments with little or no latency after the photolysis flash (Karpen, Zimmerman, Stryer, and Baylor, 1988). Alternatively, if Ins[1,4,5]P_3 opens a conductance through a cascade of reactions, a significant latency after photolysis of caged Ins[1,4,5]P_3 might be expected.

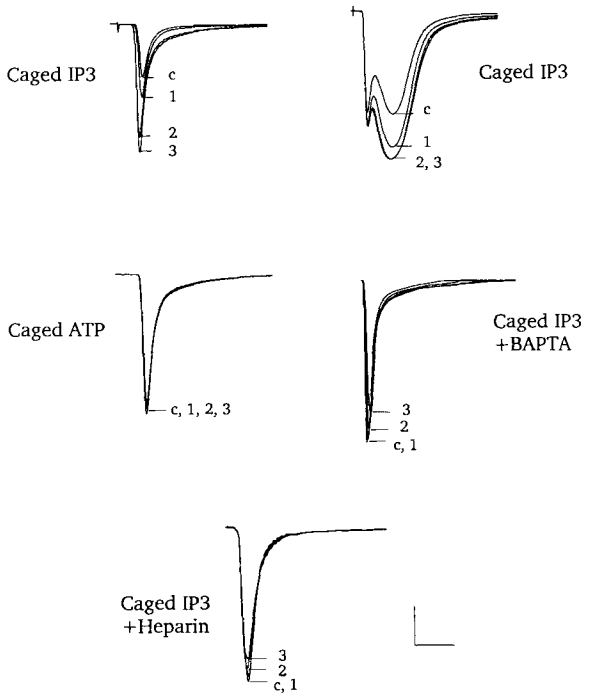

FIGURE 11. Effect of flash photolysis of caged Ins[1,4,5]P_3 in photoreceptors in which phototransduction was largely uncoupled. Each cell was bathed for 10 min in 200 mM hydroxylamine while being intensely illuminated, and was then rinsed and allowed to recover in the dark for 45 min. The cells were impaled with a microelectrode containing 25 mM GDPβS with or without heparin or BAPTA. After injecting 6–20 pl of drug solution, the photoreceptors were impaled with a second microelectrode containing the caged compound (10 mM). The voltage clamp response to discharge of the flashlamp was recorded once every 5 min until a stable response amplitude was recorded (usually within three to five flashes). Caged compound was then injected (usually three to five injections during each interflash interval) and subsequent responses recorded. With this procedure, more cumulative caged InsP_3 was available intracellularly for photolysis at each successive flashlamp discharge. Each set of records contains four consecutive traces and one control (c) after injection of caged compound (1, 2, 3) recorded at 5-min intervals. The horizontal scale bar for each set of records is equal to 130 ms for all parts except caged IP3 + BAPTA, in which it is equal to 2,600 ms. The vertical scale bar is equal to 50 nA, caged IP3 (*left*); 130 nA, caged IP3 (*right*); 220 nA, caged ATP; 180 nA caged IP3 + BAPTA; and 55 nA, caged IP3 + heparin.

Caged InsP_3 was injected into isolated photoreceptors that had been previously desensitized by the combined treatment with hydroxylamine and GDPβS. The responses to unattenuated flashlamp discharges were recorded both before and after these injections. After injections, each subsequent flash produced intracellular InsP_3 by photolysis of caged compound. Fig. 11 shows examples of voltage clamp responses recorded as a cell was injected with caged Ins[1,4,5]P_3. In 11 of 13 cells that produced stable responses before injection, the response amplitude after injecting caged

Ins[1,4,5]P_3 increased to 203.5 ± 992.% (mean ± SD for maximal change, range 105–377%) of the amplitude before injection. The photocurrent latency was not significantly different after intracellular injection of caged Ins[1,4,5]P_3 (36.4 ± 2.0 ms control, 38.0 ± 2.3 ms with caged Ins[1,4,5]P_3, mean ± SEM); however, occasionally the photocurrent latency was shorter after injection of caged Ins[1,4,5]P_3, as for the example shown in Fig. 11 at the top left. In 2 of the 11 cells that had larger amplitude photocurrents after injection of caged Ins[1,4,5]P_3, the photocurrent had a secondary peak (before and after injection). An example of this is shown in the top right of Fig. 11. A similar phenomenon has been reported in "current clamp" records from untreated ventral photoreceptors (Maaz, Nagy, Stieve, and Klomfass, 1981).

Caged ATP was used as a control for effects due to release of the nitrosoacetophenone "cage" and repetitive UV flashes (Fig. 11). Unlike the findings with photolysis of caged Ins[1,4,5]P_3, the response amplitude after photolysis of intracellularly injected caged ATP was not increased (82.7 ± 18.3% of the amplitude before injection, mean ± SD for $n = 7$ cells; range 59–110%).

To determine whether the increased amplitude of the photocurrent after injection of caged Ins[1,4,5]P_3 was due to release of intracellular calcium stores, caged Ins[1,4,5]P_3 was injected into photoreceptors that had previously been injected with either the calcium chelator BAPTA or heparin, an antagonist of Ins[1,4,5]P_3-induced calcium release (Nilsson, Zwiller, Boynton, and Berggren, 1988). For these experiments, 300 mM BAPTA or 100 mg/mL heparin was coinjected with GDPβS during the desensitization protocol. Examples of experiments of this type are illustrated in Fig. 11. The response amplitude with flash photolysis of injected caged Ins[1,4,5]P_3 into BAPTA-loaded cells was 81.9 ± 10.9% for seven cells (mean ± SD, range 60–94% of control amplitude). The response amplitude with flash photolysis of injected caged Ins[1,4,5]P_3 into heparin-loaded cells was 79.0 ± 11.0% for four cells (mean ± SD, range 64–93% of control amplitude). None of the cells preloaded with either BAPTA or heparin had an increased photocurrent amplitude with photolysis of caged Ins[1,4,5]P_3.

DISCUSSION

The experiments described in this paper were undertaken on the premise that phototransduction in *Limulus* photoreceptors is mediated by a cascade of reactions. The evidence for this premise, although indirect, is compelling. A dark-adapted *Limulus* photoreceptor has the ability to produce a large signal amplification; activation of a single rhodopsin molecule can produce in excess of 1 nA of inward current, corresponding to the opening of ~1,000 ion channels. Analogously, other transduction cascades provide amplification in each of several steps, thus generating a large overall amplification (β-adrenergic receptor activation–cAMP cascade: Levitzki, 1988; cGMP cascade in vertebrate photoreceptors: Pugh and Cobbs, 1986; Stryer, 1986; Chabre and Deterre, 1989). In addition, a flash of light elicits a response that develops after a delay; the rising phase of the response has been modeled by a linear cascade of 10 first-order reactions (Fuortes and Hodgkin, 1964). This delay is ~10 ms (at 22°C) for a bright flash that activates essentially all of the rhodopsin molecules in a cell.

Uncoupling Phototransduction

Hydroxylamine, when superfused in the presence of intense illumination, reduces the quantum efficiency of the phototransduction cascade in *Limulus* photoreceptors by greater than two orders of magnitude. Several observations suggest that the basis for this effect is that hydroxylamine specifically inactivates the initial step in the phototransduction cascade, i.e., excitation of rhodopsin by light. First, hydroxylamine treatment was completely ineffective in darkness. A nonspecific chemical action of hydroxylamine would be expected to be similar in the presence and absence of illumination. Second, the ERP was abolished after treatment with hydroxylamine plus illumination; the ERP is an electrical signal arising within activated rhodopsin molecules themselves. Third, the shape of the stimulus–response relation was qualitatively normal in voltage clamped photoreceptors after treatment with hydroxylamine plus light. The effect of the treatment was a simple translation of the stimulus–response relation by several log units toward brighter stimulus intensities. A nonspecific action of hydroxylamine on multiple steps of the cascade might be expected to produce a change in the shape as well as the location of the stimulus–response relation on the intensity axis. Fourth, the waveforms of receptor potentials and photocurrents were qualitatively normal in cells after treatment with hydroxylamine plus light. This latter result suggests that components of the phototransduction cascade distal to rhodopsin activation, including ion channels, are functionally intact after hydroxylamine treatment. These observations are consistent with the idea that hydroxylamine reacts with and removes the chromophore (presumably 11-*cis* retinal) from *Limulus* rhodopsin, thereby inactivating it.

Hydroxylamine is highly reactive with free retinal, forming retinal oximes. However, *Limulus* rhodopsin and metarhodopsin are thermally stable (Lisman and Sheline, 1976); i.e., free retinal is not liberated after illumination. The chemical mechanism of the reaction of hydroxylamine with *Limulus* rhodopsin may therefore involve a direct attack (facilitated by illumination) by hydroxylamine on the Schiff base linkage of the chromophore to opsin. A similar chemical mechanism has been postulated for the reaction of hydroxylamine (in the dark) with the visual pigment of green rods (Dartnall, 1967). Alternatively, it may be postulated that a photopigment intermediate between rhodopsin and metarhodopsin is thermally unstable. As a result, the Schiff base linkage between retinal and opsin may break momentarily and reform spontaneously after a more stable opsin conformation is attained. Hydroxylamine might then react with the transiently free retinal. The results presented in this paper do not discriminate between these two postulates.

Injection of GDPβS was chosen as an adjunct to hydroxylamine treatment to uncouple more completely the phototransduction cascade in *Limulus* ventral photoreceptors. The results obtained in our study are similar to previous work that demonstrated that GDPβS shifted the stimulus–response relation to higher intensities by several orders of magnitude (Bolsover and Brown, 1982; Fein, 1986) largely, if not entirely, through a reduction in the quantum efficiency of phototransduction (Kirkwood et al., 1989). The reduction in quantum efficiency was proposed to arise from the failure of many activated rhodopsin molecules to couple to functional G proteins (Kirkwood et al., 1989).

Two new findings reported in our study are that GDPβS injection (*a*) increases the slope of the stimulus–response relation and (*b*) produces a nonlinear relationship between latency and log(stimulus intensity) in ventral photoreceptors. Both observations suggest that intracellular GDPβS either induces or uncovers a supralinear process in the phototransduction cascade. For a hypothetical example of the former case, GDPβS-bound G proteins might interact with activated rhodopsin molecules with abnormally high stoichiometry; i.e., multiple binding events with activated rhodopsin molecules might be required to activate GDPβS-bound G proteins. We are aware of no precedent for this hypothetical mechanism.

For a hypothetical example of the latter case, supralinearity might result from depression of the sensitivity of the cascade such that a supralinear mechanism, either positive feedback or feedforward, that normally occurs in the region of saturation of the stimulus–response relation may now occur in the linear region of the stimulus–response relation. Such a mechanism may explain a similar phenomenon produced by depleting the photoreceptor of calcium ions (Bolsover and Brown, 1985); calcium depletion caused a reduction in sensitivity and a supralinear stimulus–response relation. Moreover, at moderate light intensities, the current during a prolonged stimulus was initially small but became suddenly large after a delay. This delayed increase in the amplification of the cascade was accompanied by a rise in Ca_i. These findings were interpreted to indicate that (at least in calcium-depleted cells) Ca_i increased the efficiency of some step, or steps, in the cascade.

The combination of GDPβS injection and hydroxylamine bleaching reduces the quantum efficiency of *Limulus* ventral photoreceptors by a mean factor (~870,000) that is close to the product of the mean reduction factors for either treatment alone (~350,000). This result is consistent with each treatment acting by an independent mechanism.

Flash Photolysis of Caged Ins(1,4,5)P_3

The possibility that Ins(1,4,5)P_3 directly gates an ion channel in the plasma membrane of *Limulus* ventral photoreceptors appears unlikely because the conductance increase induced by Ins(1,4,5)P_3 injection is largely blocked by calcium chelators (Rubin and Brown, 1985; Payne et al., 1986*b*). However, injected Ins(1,4,5)P_3 may not have access to plasma membrane channels due to a metabolic barrier of 5-phosphatase activity between the site of injection and the membrane. Intracellular calcium ions released at the site of injection of Ins(1,4,5)P_3 might diffuse to the plasma membrane and activate a phospholipase C; thus, injection of Ins(1,4,5)P_3 might stimulate the production of Ins(1,4,5)P_3 at the plasma membrane at a site distant from the site of injection. Diffusion of calcium ions would not be affected by a metabolic barrier of 5-phosphatase but would be altered by calcium chelators. This alternative proposal can be examined by the use of caged Ins(1,4,5)P_3. Caged Ins(1,4,5)P_3 is metabolically inert (Walker, Somlyo, Goldman, Somlyo, and Trentham, 1987). Therefore, flash photolysis of caged Ins(1,4,5)P_3 should produce a concentration jump of Ins(1,4,5)P_3 at the plasma membrane of injected photoreceptors independent of the existence of a metabolic barrier of 5-phosphatase. A current would be predicted to develop virtually instantaneously after the photolysis flash if

Ins(1,4,5)P_3-gated channels are present in the plasma membrane and channel activation kinetics are rapid.

The assumption that activation kinetics would be rapid for Ins(1,4,5)P_3-gated channels is suggested by data for other ligand-gated channels. The association rate constants for the acetylcholine receptor channel (reviewed in Steinbach, 1989), the cGMP-gated channel of vertebrate photoreceptors (Karpen et al., 1988), and the Ins(1,4,5)P_3-gated calcium release channel of rat basophilic leukemia cells (Meyer, Wensel, and Stryer, 1990) are close to the diffusion-limited encounter rate, $\sim 10^8$ s^{-1}. In addition, voltage-jump relaxation and flash photolysis studies suggest that the intramolecular transition from the fully liganded closed state to the open state is < 100 μs for both the acetylcholine receptor channel (reviewed in Lester and Nerbonne, 1982) and the cGMP-gated channel (Karpen et al., 1988). The results presented in our paper do not support the prediction that a jump in the concentration of IP_3 produced by flash photolysis of caged IP_3 would induce a virtually instantaneous current. Instead, the latency of the residual current before injection of the caged compound was not significantly different than the latency of current recorded after injection and photolysis of the caged compound.

The increased amplitude of flash-evoked voltage clamp currents recorded after intracellular injection of caged Ins(1,4,5)P_3 probably depends on Ins(1,4,5)P_3-induced release of calcium from intracellular stores. This interpretation is supported by blockade of the effect by prior intracellular injection of herarin or BAPTA, which tend to block binding of Ins(1,4,5)P_3 or changes in Ca_i, respectively. The nitrosoacetophenone released intracellularly by flash photolysis of caged Ins(1,4,5)P_3 is unlikely to cause the effect because the same product of photolysis is released from caged ATP. In addition, potentiation of the current amplitude is probably not a direct result of repetitive flash exposure as flash photolysis of caged ATP does not produce the effect; changes in concentration of intracellular ATP in ventral photoreceptors are not correlated with changes in the responsiveness to light (Rubin and Brown, 1988). The observed latency of the flash-evoked current after intracellular injection of caged Ins(1,4,5)P_3 was infrequently (4 of 13 cells) shorter than control latency (e.g., the records in the top left of Fig. 11). This infrequent finding might be expected for two reasons. First, calcium iontophoresis into ventral photoreceptors reduces the latency of the current evoked by a flash of light (Brown and Lisman, 1975). Second, a concentration jump of Ins(1,4,5)P_3 produced by photolysis might be expected to by-pass several steps of the cascade activated by light. The similarity in the average latencies of the flash-evoked photocurrents before and after injecting caged Ins(1,4,5)P_3 suggests that the time course of the increase in Ca_i induced by photolysis of caged Ins(1,4,5)P_3 or by excitation of rhodopsin is comparable.

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