

# Timing of $\text{Ca}^{2+}$ Release from Intracellular Stores and the Electrical Response of *Limulus* Ventral Photoreceptors to Dim Flashes

Richard Payne and James Demas

From the Department of Biology, University of Maryland, College Park, Maryland 20742

**abstract** Light-induced release of  $\text{Ca}^{2+}$  from stores in *Limulus* ventral photoreceptors was studied using confocal fluorescence microscopy and the  $\text{Ca}^{2+}$  indicator dyes, Oregon green-5N and fluo-4. Fluorescence was collected from a spot within 4  $\mu\text{m}$  of the microvillar membrane. A dual-flash protocol was used to reconstruct transient elevations of intracellular free calcium ion concentration ( $\text{Ca}_i$ ) after flashes delivering between 10 and  $5 \times 10^5$  effective photons. Peak  $\text{Ca}_i$  increased with flash intensity to  $138 \pm 76 \mu\text{M}$  after flashes delivering  $\sim 10^4$  effective photons, while the latent period of the elevation of  $\text{Ca}_i$  fell from  $\sim 140$  to 21 ms. The onset of the light-induced elevation of  $\text{Ca}_i$  was always highly correlated with that of the receptor potential. The time for  $\text{Ca}_i$  to exceed 2  $\mu\text{M}$  was approximately equal to that for the receptor potential to exceed 8 mV (mean difference;  $2.2 \pm 6.4$  ms).  $\text{Ca}_i$  was also measured during steps of light delivering  $\sim 10^5$  effective photons/s to photoreceptors that had been bleached with hydroxylamine so as to reduce their quantum efficiency. Elevations of  $\text{Ca}_i$  were detected at the earliest times of the electrical response to the steps of light, when a significant receptor potential had yet to develop. Successive responses exhibited stochastic variation in their latency of up to 20 ms, but the elevation of  $\text{Ca}_i$  and the receptor potential still rose at approximately the same time, indicating a shared process generating the latent period. Light-induced elevations of  $\text{Ca}_i$  resulted from  $\text{Ca}^{2+}$  release from intracellular stores, being abolished by cyclopiazonic acid (CPA), an inhibitor of endoplasmic reticulum  $\text{Ca}^{2+}$  pumps, but not by removal of extracellular  $\text{Ca}^{2+}$  ions. CPA also greatly diminished and slowed the receptor potential elicited by dim flashes. The results demonstrate a rapid release of  $\text{Ca}^{2+}$  ions that appears necessary for a highly amplified electrical response to dim flashes.

**key words:** cyclopiazonic acid • horseshoe crab • fluorescent indicator dye • receptor potential • phototransduction

## INTRODUCTION

Microvillar photoreceptors of invertebrates use the phosphoinositide cascade to couple the absorption of light by rhodopsin to the activation of ion channels in the microvillar plasma membrane (Ranganathan et al., 1995; Dörlöchter and Stieve, 1997). The phosphoinositide cascade produces several diffusible messengers, including inositol (1,4,5) trisphosphate ( $\text{InsP}_3$ ),<sup>1</sup> diacylglycerol, and  $\text{Ca}^{2+}$  ions, the latter being released from intracellular stores by the action of  $\text{InsP}_3$  (Berridge and Irvine, 1989). In this paper, we describe a rapid light-induced release of  $\text{Ca}^{2+}$  from intracellular stores in the giant ventral photoreceptor of *Limulus polyphemus*, which is necessary for a highly amplified electrical response to dim flashes and which limits temporal acuity.

The role of light-induced  $\text{Ca}^{2+}$  release in excitation of invertebrate photoreceptors is uncertain and may depend on the species studied. In *Drosophila* photoreceptors, light-induced release of  $\text{Ca}^{2+}$  has been observed

(Arnon et al., 1997), but the light-induced elevation of intracellular free calcium ion concentration ( $\text{Ca}_i$ ) results mostly from an influx through the light-sensitive channels, which may therefore be activated by a messenger other than  $\text{Ca}^{2+}$  ions or  $\text{InsP}_3$  (Ranganathan et al., 1994; Peretz et al., 1994; Acharya et al., 1997; Chyb et al., 1999). For *Limulus* ventral photoreceptors, however, light-induced elevation of  $\text{Ca}_i$  is dominated by the release of  $\text{Ca}^{2+}$  from intracellular stores (Brown and Blinks, 1974), which may be necessary to maintain the sensitivity of visual excitation (Bolsover and Brown, 1985). There is also evidence that release of  $\text{Ca}^{2+}$  excites *Limulus* ventral photoreceptors. Intracellular pressure injection of  $\text{InsP}_3$  or  $\text{Ca}^{2+}$  ions in darkness activates a conductance with a reversal potential and relative permeability to sodium ions similar to that of the light-sensitive conductance (Fein et al., 1984; Brown et al., 1984; Payne et al., 1986). Rapid release of  $\text{Ca}^{2+}$  by flash photolysis of intracellular caged  $\text{InsP}_3$  or caged  $\text{Ca}^{2+}$  activates this conductance within a few milliseconds (Ukhanov and Payne, 1997). Pharmacological blockade of light-induced  $\text{Ca}^{2+}$  release greatly diminishes the electrical response to dim or moderately intense light flashes and slows the onset of the electrical response at all intensities (Payne and Fein, 1986; Frank and Fein, 1991; Shin et al., 1993; Nagy and Contzen, 1997; Johnson et al., 1998). Cyclopiazonic acid

Address correspondence to Richard Payne, Department of Biology, University of Maryland, College Park, MD 20742. Fax: 301-314-9358; E-mail: rp12@umail.umd.edu

<sup>1</sup>Abbreviations used in this paper: ASW, artificial sea water; CPA, cyclopiazonic acid; ER, endoplasmic reticulum;  $\text{InsP}_3$ , inositol (1,4,5) trisphosphate; R-lobe, rhabdomeral lobe.

(CPA), an agent that depletes intracellular  $\text{Ca}^{2+}$  stores (Mason et al., 1991), eliminates the highly amplified response to single quanta (Dorlöchter et al., 1999).

Measurement of  $\text{Ca}^{2+}$  at spots within a few microns of the photosensitive membrane by confocal fluorescence microscopy has permitted the detection of elevations of  $\text{Ca}_i$  that precede the electrical response to very intense focal stimulation (Ukhanov and Payne, 1995). We now investigate similarly rapid elevations of  $\text{Ca}_i$  to moderate and dim flashes, for which the pharmacological evidence of a role for  $\text{Ca}^{2+}$  release in excitation is most convincing.

## MATERIALS AND METHODS

### *Preparation of Photoreceptor Cells, Electrical Recording and Solutions*

Ventral optic nerves were dissected as described by Clark et al. (1969) and placed in artificial sea water (ASW) containing (mM): 435 NaCl, 10 KCl, 20  $\text{MgCl}_2$ , 25  $\text{MgSO}_4$ , 10  $\text{CaCl}_2$ , 10 HEPES, pH 7.0. 0 Ca-ASW was prepared as for ASW, but with no  $\text{CaCl}_2$  and 1 mM EGTA added. For some experiments, cells were chemically bleached to reduce their quantum efficiency (Faddis and Brown, 1992). A bleaching solution containing (mM) 200 hydroxylamine chloride (Sigma-Aldrich), 235 NaCl, 10 KCl, 20  $\text{MgCl}_2$ , 25  $\text{MgSO}_4$ , 10  $\text{CaCl}_2$ , 10 HEPES, adjusted to pH 6.5 with 10 N NaOH and cooled to 4°C. Nerves were rinsed with 25 ml of bleaching solution, and then immediately exposed to 20 min of intense white light at 4°C. Nerves were then washed five times at 10-min intervals with 50 ml of ASW at 4°C. Nerves were bleached in this manner four times with at least 8 h between bleaches, with the intervals spent at 4°C in a modified culture medium (Kass et al., 1988, modified from Bayer and Barlow, 1978). This treatment reduced the quantum efficiency of the bleached photoreceptors by  $\sim 3 \log_{10}$  U. Cyclopiazonic acid (Calbiochem) was stored as a 25-mM stock solution in DMSO, which was diluted to 100  $\mu\text{M}$  (0.4% DMSO) in ASW immediately before use.

Membrane potential was recorded through the micropipette used for pressure-injecting dye solutions. For some experiments, cells were impaled with a second electrode filled with 3 M KCl, through which current was supplied from a voltage-clamp amplifier (Axoclamp2A; Axon Instruments, Inc.).

### *Fluorescence Microscopy and Calibration of Illumination*

Ventral nerves were viewed with an LSM 410 laser-scanning, inverted, confocal microscope (Carl Zeiss, Inc.) equipped with a 488-nm Ar-Kr laser (Uniphase Inc.) attenuated 300-fold and focused through a Neofluar  $\times 40/0.75$  objective lens (for details, see Ukhanov and Payne, 1995). A high-speed shutter (Uniblitz No. 26L; Vincent Associates) was placed in the path of the laser beam to control the timing and duration of flashes. To generate a dim flash, followed by a bright one, Kodak Wratten neutral density filters were placed in the path of the laser, attached to the mechanism of a second high-speed shutter that was capable of removing the filters from the light path within 10 ms. Illumination not subject to this filtering is referred to in the text as "unattenuated."

Photoreceptors were impaled with micropipettes containing 500  $\mu\text{M}$  of the fluorescent  $\text{Ca}^{2+}$  indicator dyes, fluo-4 or Oregon green-5N (Molecular Probes, Inc.; dissociation constants determined to be 2 and 18  $\mu\text{M}$ , respectively, in 400 mM KCl, 10 mM MOPS, pH 7.0) in a carrier solution containing 100 mM potassium aspartate, 10 mM HEPES, pH 7.0. 5–15 pressure injections of 1–10 pl were delivered into the cells. After loading the cells with dye, the site of origin of the  $\text{Ca}^{2+}$  release was determined.

Photoreceptors were first viewed in the transmission mode of the microscope so as to determine the likely position of the light-sensitive rhabdomeral lobe (R-lobe). The position of the R-lobe was then confirmed by performing a line scan of fluorescence along the longitudinal axis of the cell. If the R-lobe was correctly identified, then, upon opening the shutter covering the laser beam, a delayed wave of increased fluorescence spread into the cell from the point at which the scanning line intersected the edge of the R-lobe (Ukhanov and Payne, 1995). To reduce noise, scans were spatially filtered, limiting to 4  $\mu\text{m}$  the resolution of the site at which light-induced  $\text{Ca}^{2+}$  release originated. Subsequent measurements of the time course of  $\text{Ca}^{2+}$  release were made at this point of intersection at the edge of the R-lobe in the microscope's spot mode, with the scanning movement of the laser beam halted.

Light intensities are expressed as "effective photons." One effective photon is the light energy required to photo-isomerize one rhodopsin molecule and so to elicit one quantal electrical response, a "quantum bump," from a dark-adapted photoreceptor (Yeandle and Spiegler, 1973). The laser beam could therefore be calibrated by counting the number of quantal events per second generated by a photoreceptor during continuous illumination when the beam was attenuated by a known amount. Using this calibration method, the unattenuated laser was estimated to deliver  $\sim 10^8$  effective photons/s to ventral photoreceptor cells that were not chemically bleached and  $\sim 10^5$  effective photons/s to chemically bleached cells.

### *Calibration of Fluorescence*

Autofluorescence from cells not injected with indicator dye was observed to be negligible. Photomultiplier records of dye fluorescence,  $F$ , from injected cells were used to determine the mean fluorescence,  $F_{\text{rest}}$ , during the latent period of the light response and the change in fluorescence,  $\Delta F = F - F_{\text{rest}}$ , during the light response. For each cell, the saturated peak fluorescence,  $F_{\text{max}}$ , during illumination by the unattenuated laser was also recorded. On the assumption that the dye is saturated by the peak elevation of  $\text{Ca}_i$  that follows flashes delivered by the unattenuated laser (see results),  $\text{Ca}_i$  can be estimated using the standard equation (Grynkiewicz et al., 1985):

$$[\text{Ca}^{2+}] = K_d \cdot (F - F_{\text{min}}) / (F_{\text{max}} - F), \quad (1)$$

where  $K_d$  is the dissociation constant of the dye and  $F_{\text{min}}$  is the fluorescence of the indicator with no  $\text{Ca}^{2+}$  bound. Two assumptions were adopted to obtain  $F_{\text{min}}$ . For the low affinity indicator, Oregon green-5N, the dye was assumed to bind negligible  $\text{Ca}^{2+}$  at resting  $\text{Ca}_i$ , but to have a comparatively significant calcium-independent fluorescence.  $F_{\text{min}}$  is then equal to  $F_{\text{rest}}$ . In terms of the fluorescence changes recorded following illumination, Eq. 1 becomes Eq. 2:

$$[\text{Ca}^{2+}] = K_d \cdot \Delta F / (\Delta F_{\text{max}} - \Delta F) \quad (2)$$

or Eq. 3:

$$[\text{Ca}^{2+}] = K_d \cdot (\Delta F / \Delta F_{\text{max}}) / (1 - \Delta F / \Delta F_{\text{max}}), \quad (3)$$

where  $\Delta F_{\text{max}} = F_{\text{max}} - F_{\text{rest}}$ .

For the high affinity indicator, fluo-4, calcium-independent fluorescence was assumed to be negligible compared with the fluorescence due to resting  $\text{Ca}_i$ .  $F_{\text{min}}$  is then equal to zero. In terms of the fluorescence recorded after illumination, Eq. 1 becomes Eq. 4:

$$[Ca^{2+}] = K_d \cdot F / (F_{\max} - F). \quad (4)$$

The protocol used for reconstructing elevations of  $Ca_i$  after dim flashes is similar to that adopted by Gray-Keller and Detwiler (1996) in their study of vertebrate rod photoreceptors, with the exception that all of the measurements for a given dim flash intensity could be made on a single cell, since photobleaching of visual pigment does not occur in *Limulus* photoreceptors (Lisman and Sheline, 1976). The method takes advantage of the latent period that precedes the elevation of  $Ca_i$  and electrical response. Dim flashes do not elicit sufficient dye fluorescence to enable measurement of  $Ca_i$ . However, a dim flash can be paired with a subsequent bright flash delivered by the unattenuated laser. Dye fluorescence sampled during the 20–30-ms latent period of the response to the bright flash is assumed to reflect the  $[Ca^{2+}]$  that would have prevailed during the response to the dim flash alone. The latent period of the response to the bright flashes can be defined as the time at which receptor potentials following the bright flashes (Fig. 1 A, 1–5) depart significantly from the response to the dim flash alone. This deviation from the response to the dim flash occurred after the bright flash was over, so that fluorescence was only collected during the latent period.

By varying the time between dim and bright flashes, the time course of the elevation of  $Ca_i$  that followed the dim flash could be reconstructed (Fig. 1), provided adequate time for dark adaptation was allowed between the paired flashes. To extend the time-window during which measurements of  $Ca_i$  could be made, the latent period of the response to the bright flash could be extended to 70–150 ms by lowering the quantum efficiency of the cell through chemical bleaching of rhodopsin. In comparing the waveforms of the reconstructed calcium signals and the receptor potential in Figs. 2, 4, 6, and 10, it is important to note that the receptor potential shown is a representative response to the dim flash alone, recorded between presentations of the dual flashes. As Fig. 1 illustrates, there was some variation in the latency of the receptor potential from flash to flash, which becomes significant for dim flashes delivering <1,000 effective photons. This variation reduces the accuracy with which the latency of the estimated elevations of  $Ca_i$  and that of the receptor potential can be compared. Stable recordings of dye fluorescence and membrane potential were generally obtained for up to 90 min after injection of the dye. With ~10 min between dual flash presentations required for dark adaptation in unbleached photoreceptors, reconstructions of elevations of  $Ca_i$  were usually limited to a maximum of 9 or 10 dual flash presentations.

### Synchronization of Traces

Membrane potential or current traces were digitally sampled at a rate of 1 kHz and stored on a computer, simultaneously with samples of the command pulses sent to the amplifier controlling the shutter placed in front of the laser beam. The shutter command pulses stored in these records were used to synchronize the current or voltage traces with the onset of fluorescence in the digitized records of photomultiplier output stored in the confocal microscope, after allowing 1.5 ms for shutter opening, as described in Ukhonov and Payne (1995).

## RESULTS

### The Amplitude and Latency of the Elevation of $Ca_i$ Is Graded with Flash Intensity

Photoreceptors were filled with the low affinity indicator, Oregon green-5N. A dual flash protocol (see materials and methods) was used to reconstruct changes in

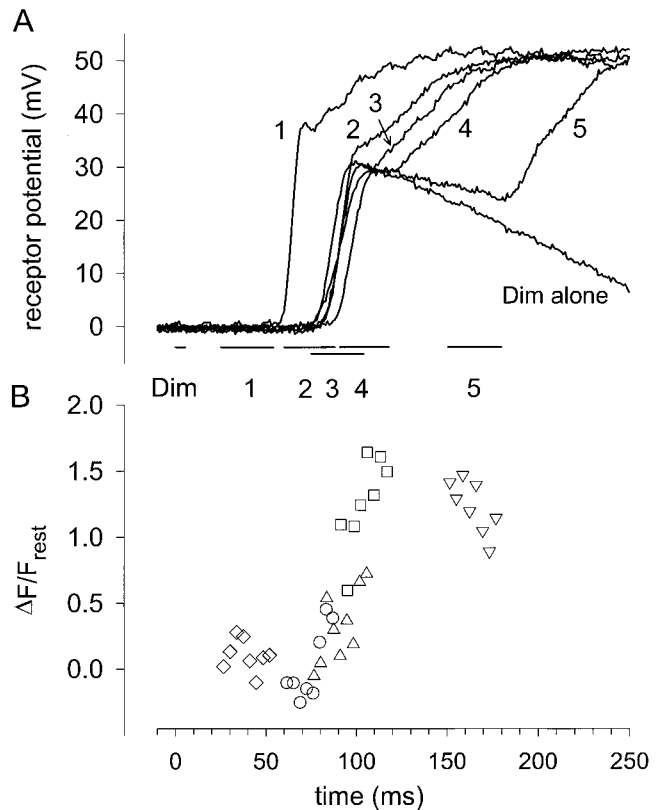


Figure 1. (A) Superimposed receptor potentials recorded after five pairs of dim and then bright flashes and after one dim flash alone. The flashes were delivered at the times indicated by the stimulus monitors below the membrane potential traces. For each pair, a dim flash (5-ms duration, delivering ~500 effective photons) was followed after a variable interval by a bright flash (1–5), delivered by the unattenuated laser (30-ms duration). After each bright flash, the receptor potentials departed after a latent period from the response to the dim flash alone and saturated at 50 mV. (B) Relative fluorescence,  $\Delta F/F_{\text{rest}}$  of Oregon green-5N (clusters of identical symbols) recorded during the latent period of the responses to the five bright flashes.

dye fluorescence after dim flashes. Photoreceptors were dark adapted between presentations of dual flashes so that bumps of 1–5 mV could be recorded. For each cell, the saturated peak fluorescence,  $F_{\max}$ , was also recorded during illumination by a step of light from the unattenuated laser. After dim flashes, transient increases in dye fluorescence could be reconstructed that increased in peak amplitude with increasing flash intensity (Fig. 2). The peak amplitude of the fluorescence saturated at  $F_{\max}$  for flash energies delivering  $>5 \times 10^4$  effective photons (Fig. 3 A). This saturation is assumed to reflect that of the  $Ca^{2+}$ -indicator dye rather than the value of  $Ca_i$ , since peak  $Ca^{2+}$  concentrations exceeding 150  $\mu\text{M}$  have been recorded in response to a step of illumination by the unattenuated laser beam using a lower affinity dye, Calcium-Green 5N ( $K_d = 67 \mu\text{M}$ ; Ukhonov et al., 1995). On this assumption and also assuming that the dye

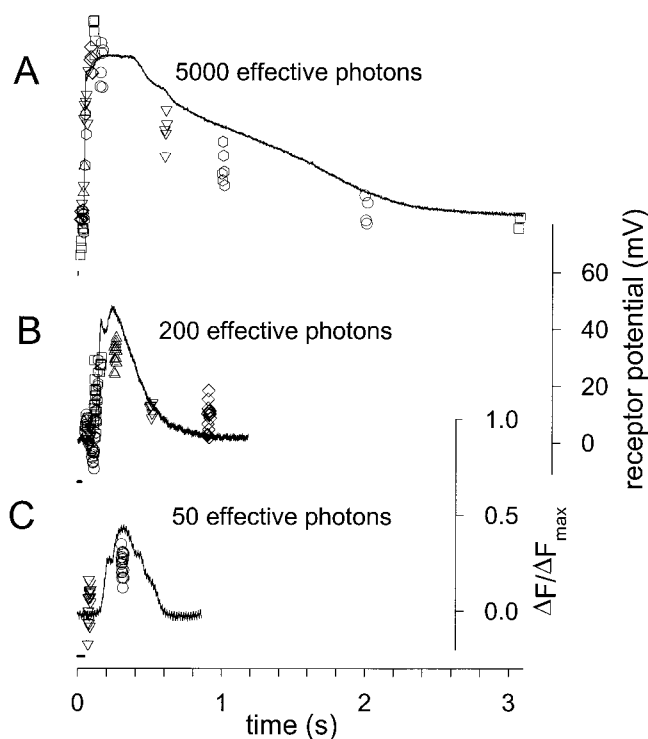


Figure 2. Receptor potentials (solid lines) and Oregon green-5N fluorescence, (symbols) recorded after flashes that delivered 5,000 (A), 200 (B), and 50 (C) effective photons. Dye fluorescence is shown as the ratio of the fluorescence increase that followed a dim flash,  $\Delta F$ , to the peak fluorescence increase,  $\Delta F_{\max}$ , observed during a step of unattenuated laser illumination delivered to the same confocal spot. Bars below each set of traces indicate the timing of the dim flashes, 5-, 20-, and 50-ms duration and 2, 4, and 5 log<sub>10</sub> U attenuation, respectively.

binds negligible  $\text{Ca}^{2+}$  in dark-adapted cells at rest (see materials and methods), the peak amplitudes of the light-induced elevations of  $\text{Ca}_i$  were calibrated (Fig. 3 B). Estimated peak  $\text{Ca}_i$  rose approximately as light intensity raised to the power 0.6, a much more steep relationship than that observed for  $\text{Ca}_i$  during prolonged background illumination, which increases as light intensity raised to the power 0.1–0.2 (Levy and Fein, 1985; Ukhonov et al., 1995), but comparable with that observed by Oberwinkler and Stavenga (1998) during sustained illumination of blowfly photoreceptors. The dimmest flashes for which elevations of  $\text{Ca}_i$  could be reliably detected at the peak of the receptor potential delivered  $\sim 50$  effective photons and generated estimated peak increases in  $\text{Ca}_i$  of  $5.0 \pm 1.8 \mu\text{M}$  (SEM,  $n = 7$ ), or  $\sim 300$  free  $\text{Ca}^{2+}$  ions per effective photon in an estimated confocal measurement volume of  $\sim 5 \mu\text{m}^3$ .

#### *The Rising Edges of the Receptor Potential and of the Elevation of $\text{Ca}_i$ Are Highly Correlated in Time*

For flashes delivering  $<200$  effective photons, the elevation of  $\text{Ca}_i$  recorded using Oregon green-5N was too

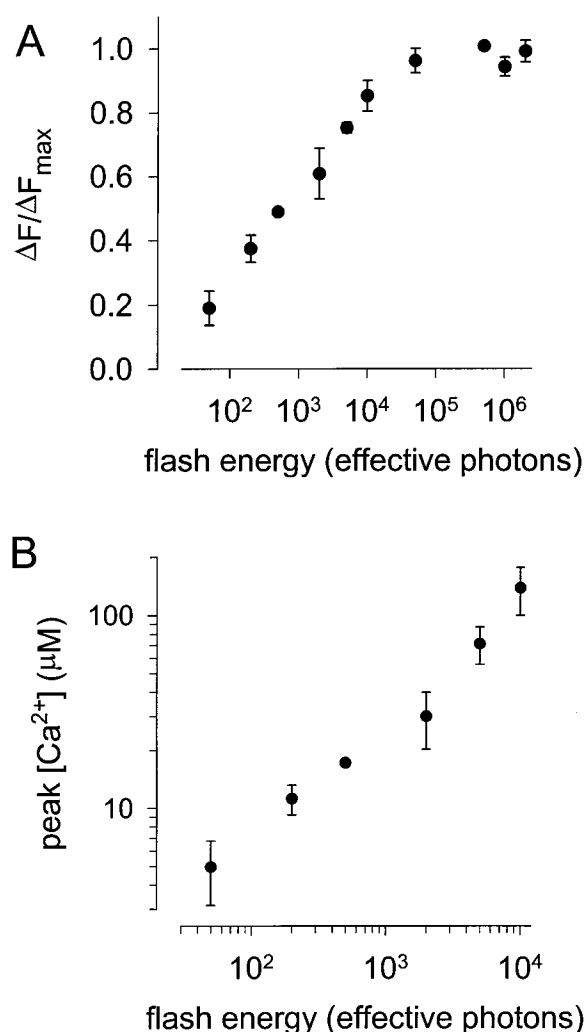


Figure 3. (A) The light-induced increase in Oregon green-5N fluorescence at the peak of each response (mean  $\pm$  SEM) is plotted against the flash energy. The fluorescence increase,  $\Delta F$ , is shown relative to the peak fluorescence increase,  $\Delta F_{\max}$ , observed during a step of unattenuated laser illumination to the same confocal spot. (B) Light-induced increases in intracellular free calcium concentration,  $\text{Ca}_i$ , (mean  $\pm$  SEM), calculated from the data of B using Eq. 3.

small to reliably reconstruct its time course, and only the elevation of  $\text{Ca}_i$  at the peak of the receptor potential was sampled (but see below for fluo-4 signals). For more intense flashes, the time course of the initial elevation of  $\text{Ca}_i$  could be reconstructed (Fig. 4). The peak receptor potentials after these flash intensities were all close to saturation, but their latencies greatly differed. Despite this difference in response latency, the rising edge of the reconstructed elevation of  $\text{Ca}_i$  was always approximately coincident with that of a representative receptor potential. For the dimmest flashes, the latencies of the response varied considerably from cell to cell (Fig. 5 A), but for any given cell, the electrical signal and elevation of  $\text{Ca}_i$  were approximately coincident. As

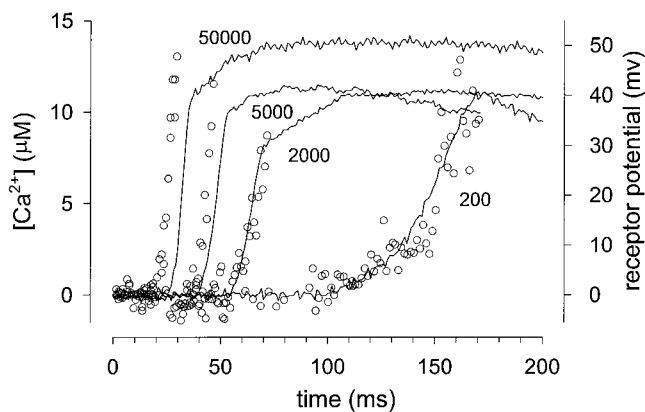


Figure 4. Rising phases of receptor potentials (solid lines) and reconstructed elevations of  $\text{Ca}_i$  ( $\circ$ ) recorded from four different cells after flashes that delivered 50,000, 5,000, 2,000, and 200 effective photons. The flashes, 5-, 5-, 20-, and 20-ms duration; 1, 2, 3, and 4  $\log_{10}$  U attenuation, respectively, were delivered at the origin of the time axis.

a measure of the latency of the elevation of  $\text{Ca}_i$ , we chose the time,  $T_{c, \text{Ca}^{2+}}$ , taken for  $\text{Ca}_i$  to exceed a criterion of 2  $\mu\text{M}$ , the smallest increase that could be reliably distinguished in individual reconstructions when using Oregon green-5N as the  $\text{Ca}^{2+}$  indicator dye.  $T_{c, \text{Ca}^{2+}}$  fell from an average of 141 ms for four cells illuminated by the dimmest flash to 21 ms as flash intensity increased (Fig. 5 A). For this range of response latencies and light intensities,  $T_{c, \text{Ca}^{2+}}$  was approximately equal to the time,  $T_{c, \text{mV}}$ , taken to exceed an 8-mV criterion receptor potential (Fig. 5 B). The relationship could be well described ( $r^2 = 0.987$ ;  $n = 13$ ) by the equation:

$$T_{c, \text{mV}} = T_{c, \text{Ca}^{2+}} + \Delta t, \quad (5)$$

with  $\Delta t$  equal to  $2.2 \pm 6.4$  ms. This time difference is not significant ( $P > 0.05$ ).

For flashes delivering 50 or fewer effective photons, the change in Oregon green-5N fluorescence was insufficient to allow a detailed analysis of the time course of the response. A higher affinity indicator dye, fluo-4, was therefore used. The rising edge of the elevation of  $\text{Ca}_i$  induced by these dim flashes was also approximately coincident with the receptor potential (Fig. 6, A and B; representative of recordings from three cells).  $T_{c, \text{Ca}^{2+}}$  and  $T_{c, \text{mV}}$  were both equal to 146 ms for criteria of 2  $\mu\text{M}$  and 8 mV, respectively. In the same cell, the response to a step of illumination from the unattenuated laser beam also demonstrated coincidence of the receptor potential and the elevation of  $\text{Ca}_i$  (Fig. 6 B).  $T_{c, \text{Ca}^{2+}}$  was 30 ms, and  $T_{c, \text{mV}}$  was 29 ms. The receptor potential and elevation of  $\text{Ca}_i$  were, therefore, approximately coincident in this cell for responses to illumination that differed in intensity by five orders of magnitude.

The elevation of  $\text{Ca}_i$  and receptor potential arise

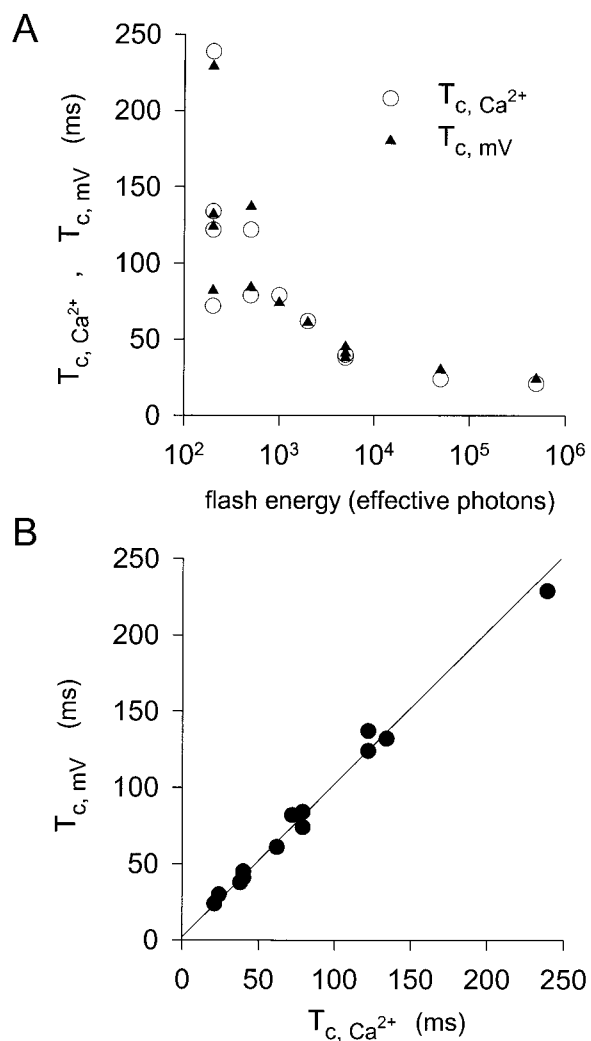


Figure 5. (A) The times taken for receptor potentials ( $T_{c, \text{mV}}$ ;  $\blacktriangle$ ) and reconstructed elevations of  $\text{Ca}_i$  ( $T_{c, \text{Ca}^{2+}}$ ;  $\circ$ ) recorded from 13 cells filled with Oregon green-5N to exceed 8 mV or 2  $\mu\text{M}$ , respectively, are plotted against the flash energy. (B) For each flash response used to create A,  $T_{c, \text{mV}}$  is plotted against  $T_{c, \text{Ca}^{2+}}$ . The solid straight line represents Eq. 5 in the text, with  $\Delta t = 2.2$  ms.

from the same stochastic process. Chemical bleaching of rhodopsin by hydroxylamine (see materials and methods) reduced the quantum efficiency of photoreceptors by  $\sim 3 \log_{10}$  U, while maintaining quantum bumps that were up to 10 mV in amplitude and of normal time course. Neither the latency of the responses delivering approximately the same number of effective photons nor the resting membrane potential was qualitatively altered by bleaching. This treatment reduced the effective intensity of the unattenuated laser from  $\sim 10^8$  to  $\sim 10^5$  effective photons/s, prolonging the latency of the response to continuous illumination from 20–30 to 70–150 ms. Because the laser illumination was unattenuated and continuous, the entire time course of an elevation of  $\text{Ca}_i$  accompanying a receptor poten-

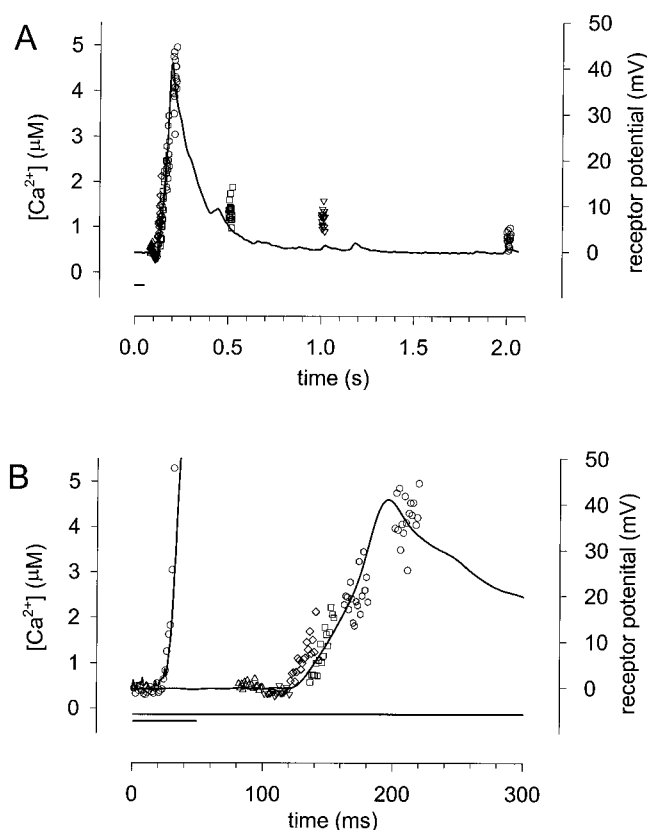


Figure 6. (A) Receptor potential (solid line) and reconstructed elevation of  $\text{Ca}_i$  (symbols) recorded after a flash that delivered  $\sim 50$  effective photons to a photoreceptor filled with fluo-4. The bar beneath the trace indicates the timing of the flash, 50-ms duration,  $5 \log_{10} U$  attenuation. (B) The rising edge of the response in A is shown on an expanded time scale, together with an estimate of  $\text{Ca}_i$  and a receptor potential recorded during a step of illumination by the unattenuated laser beam, delivering  $\sim 10^8$  effective photons/s. The bars below the traces indicate the onset and duration of the stimuli.

tial could be determined and its latency correlated with that of the receptor potential. Thus, the problem of comparing elevations of  $\text{Ca}_i$  reconstructed from many flash responses with a representative receptor potential was eliminated.

After chemical bleaching, successive receptor potentials to unattenuated step illumination by the laser exhibited variation in their latent period (Fig. 7). Variation in the latency of the receptor potential after dim flashes is thought to result from the stochastic nature of the underlying responses to single effective photons (Yeandle and Srebro, 1970; Kraemer et al., 1989). Elevations of  $\text{Ca}_i$ , measured using fluo-4, that accompanied each receptor potential showed latency variations similar to those of the receptor potential (Fig. 7). To quantitatively compare the fluctuations in latency, a criterion of either 1 or 2  $\mu\text{M}$   $\text{Ca}_i$  was selected for each of five cells so as to best match the mean value of  $T_{c, \text{Ca}^{2+}}$  to that of  $T_{c, \text{mV}}$ . The values of  $T_{c, \text{Ca}^{2+}}$  and  $T_{c, \text{mV}}$  were then com-

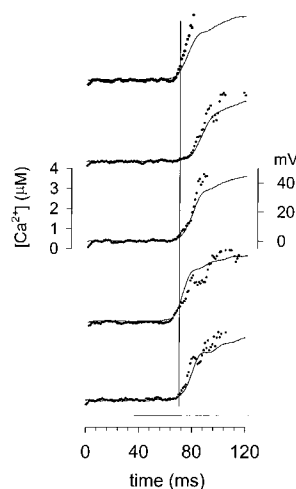


Figure 7. Receptor potentials (lines) and estimated elevations of  $\text{Ca}_i$  (●) recorded from a chemically bleached photoreceptor filled with fluo-4 during five successive continuous steps of illumination from the unattenuated laser beam, delivered at the origin of the time axis (bar below traces). The latencies of the signals vary on either side of the vertical line from flash to flash.

pared for individual flashes and were found to be highly correlated and approximately equal. For the cell of Fig. 7, their relationship could be well described ( $r^2 = 0.951$ ;  $n = 5$ ; Fig. 8 A) by Eq. 5, with  $\Delta t = 1.4 \pm 1.14$  ms. This time difference is not significant ( $P > 0.05$ ).

To compare data from all five cells, the mean values of  $T_{c, \text{mV}}$  and  $T_{c, \text{Ca}^{2+}}$  recorded from a given cell were subtracted from those of individual signals (Fig. 8 B). The resulting deviation scores of  $T_{c, \text{mV}}$  were equal to those of  $T_{c, \text{Ca}^{2+}}$  ( $r^2 = 0.829$ ). Thus, if an elevation of  $\text{Ca}_i$  rose, for example, to exceed its criterion 10 ms earlier than its mean for that cell, it was highly probable that the electrical response would also exceed its criterion 10 ms earlier than its mean. This correlation indicates that the process generating the stochastic latency is shared by both signals.

While the results so far demonstrate that the timing of the electrical and  $\text{Ca}^{2+}$  signals are highly correlated, the absolute value of  $\Delta t$  does not indicate whether the  $\text{Ca}^{2+}$  signal “leads” or “lags” the electrical response of a cell.  $\Delta t$  rather indicates the relative time for the two signals to reach criterion amplitudes and its value is therefore dependent upon the choice of those criteria. In further considering the significance of  $\Delta t$ , we noted that it varied from +1.8 to −13 ms for the five cells studied, for criterion responses of 8 mV and 2  $\mu\text{M}$ . We wished to determine whether this variation of  $\Delta t$  between cells was due to systematic differences between recordings from different cells or due to noise inherent in estimating  $\text{Ca}_i$ . Fig. 9 illustrates five responses each from two cells. To eliminate the variation in response latency from flash to flash, the time base of each step response has been normalized by subtracting  $T_{c, \text{mV}}$ . The two sets of responses show that the time course of the elevation of  $\text{Ca}_i$  relative to that of the receptor potential varies between the two cells in a systematic manner. The elevation of  $\text{Ca}_i$  was first detected  $17.3 \pm 4.5$  ms before  $T_{c, \text{mV}}$  in Fig. 9 A (arrow) and  $7.4 \pm 2.1$  ms before  $T_{c, \text{mV}}$  in B (arrow) (detec-

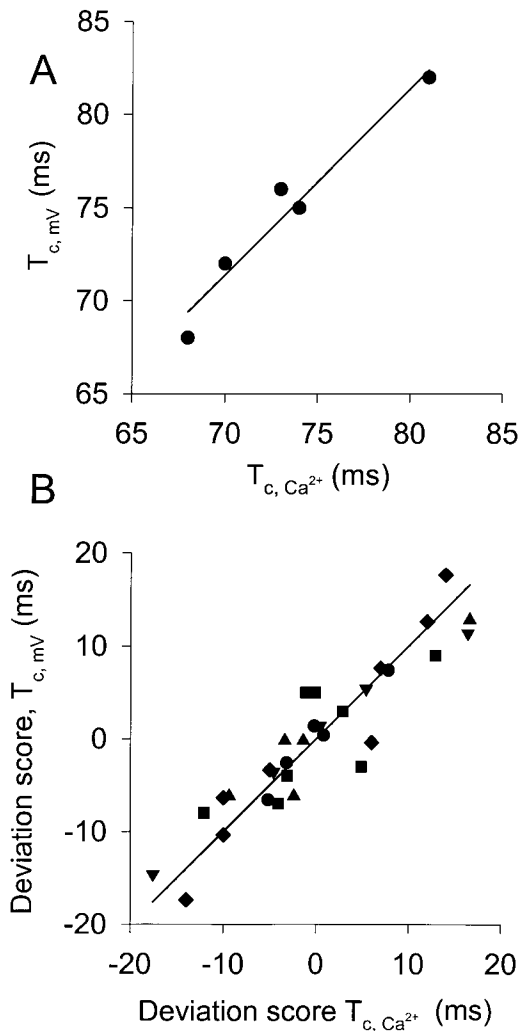


Figure 8. (A) For each of the five responses shown in Fig. 7, the times,  $T_{c, mV}$ , taken for receptor potentials to exceed 8 mV are plotted against the times,  $T_{c, Ca^{2+}}$ , taken for the elevation of  $Ca_i$  to exceed  $1 \mu M$ . The solid straight line represents Eq. 5 in the text, with  $\Delta t = 1.4$  ms. (B) To compare fluctuations in the latencies of receptor potentials and elevations of  $Ca_i$  measured in five cells filled with fluo-4,  $T_{c, mV}$  and  $T_{c, Ca^{2+}}$  for individual responses were subtracted from their means for each cell, and the resulting deviation scores were plotted against one another. The solid straight line indicates equality.

tion was based on a criterion of two successive samples of  $Ca_i$  greater than 2 SD above the initial noise level). This variation in the relative timing of the signals from cell to cell, which may arise from small displacements of the confocal spot relative to  $Ca^{2+}$  release sites (see discussion), prevents us from determining an accurate relationship between  $Ca_i$  and the receptor potential amplitude. However, it is apparent from Fig. 9 that the light-induced elevation of  $Ca_i$  is detectable at very small depolarizations. For the five cells, the mean depolarization was not significant ( $1.1 \pm 1.2$  mV;  $P > 0.05$ ) at the time the light-induced elevation of  $Ca_i$  was first detected.

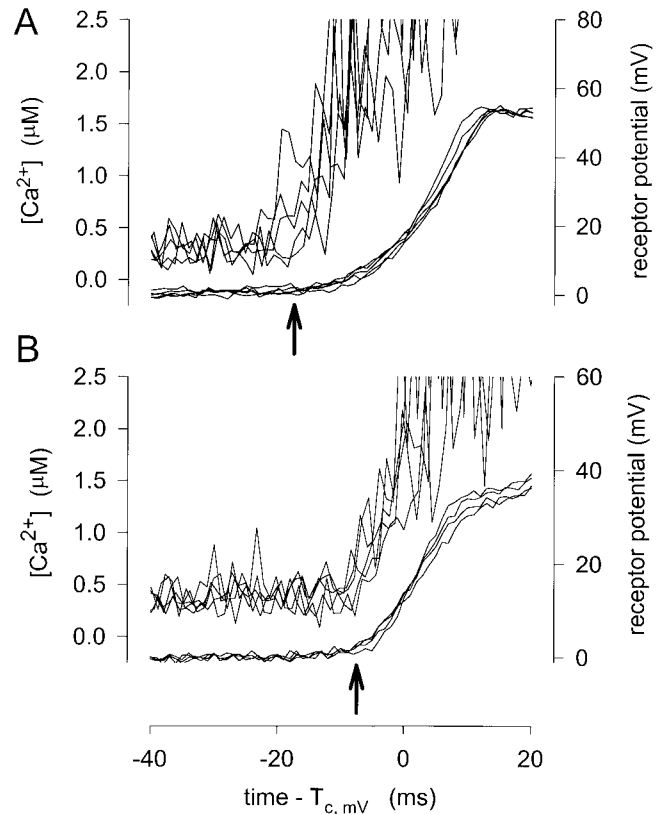


Figure 9. Superimposed elevations of  $Ca_i$  and receptor potentials (top and bottom traces, respectively, in each panel) during five successive steps of illumination delivered from the unattenuated laser beam to two cells, A and B, filled with fluo-4. To compensate for the variable latency of each response, the value of  $T_{c, mV}$  was determined for each response and was subtracted from the time base of each response. The arrows show the times when the light-induced increase of  $Ca_i$  was detectable, indicating the variation in the timing of the  $Ca^{2+}$  signal relative to that of the electrical response for these cells.

#### *Elevations of $Ca_i$ Can Be Measured to Flashes that Deliver as Few as 10 Effective Photons, When Quantal Fluctuations in Amplitude become Significant*

After chemical bleaching, the increased latency of the response to the unattenuated laser allowed a greater time "window" in which to measure the elevation of  $Ca_i$ . Also, because the flashes used to elicit dye fluorescence were less effective, the time for dark adaptation between pairs of flashes was reduced from  $\sim 10$  to  $\sim 2$  min, allowing more samples to be taken while dye fluorescence levels were stable in the cell. These improvements allowed the measurement of elevations of  $Ca_i$  in cells filled with fluo-4 that accompanied extremely dim flashes, delivering  $\sim 10$  effective photons, which demonstrated considerable stochastic variation in the amplitude and latency of individual receptor potentials (Fig. 10 A). Although the accuracy of the comparison was limited to  $\sim 20$  ms by variation in individual responses (Fig. 10 B), the first

appearance of a calcium signal was still approximately coincident with the appearance of the electrical response. In the same cell, the response to a step of illumination from the unattenuated laser beam also demonstrated coincidence of the receptor potential and the elevation of  $Ca_i$  (Fig. 10 B). Similar elevations of  $Ca_i$  were obtained from two other chemically bleached cells.

#### *The Elevation of $Ca_i$ Arises from Release of Intracellular $Ca^{2+}$ , Not Influx*

For bright steps of light, delivered by the unattenuated laser, the timing of the elevation of  $Ca_i$ , recorded from unbleached cells, relative to that of the electrical response, is unaffected by voltage clamping cells to their resting potential or by removal of extracellular  $Ca^{2+}$  (Ukhanov and Payne, 1995). We wished to confirm this result using bleached photoreceptors and for dim flashes. In hydroxylamine-treated cells that were voltage clamped to their membrane potential in darkness (typically between  $-50$  and  $-65$  mV), the elevation of  $Ca_i$  measured by fluo-4 was approximately coincident with the appearance of the photocurrent initiated by a step of illumination from the unattenuated laser beam (Fig. 11 A). Similar results were obtained in a total of five cells. Bathing the cell of Fig. 11 A in 0 Ca-ASW for 15 min reduced the initial dye fluorescence, reflecting a decrease in resting  $Ca_i$  (Bolsover and Brown, 1985; Levy and Fein, 1985), and increased the latency of both the photocurrent (Martinez and Srebro, 1976) and the elevation of  $Ca_i$ . However, the onset of the photocurrent and the elevation of  $Ca_i$  were still approximately coincident (Fig. 11 B).

Reconstruction of the complete time course of elevations of  $Ca_i$  after dim flashes delivered to unbleached cells bathed in 0 Ca-ASW was not possible because of the progressive depletion of  $Ca^{2+}$  stores by the repeated bright flashes required to measure dye fluorescence (see below). However, up to five dual flashes could be delivered without greatly affecting the magnitude or latency of successive responses, so that a portion of the rising phase of elevations of  $Ca_i$  could be reconstructed (Fig. 11 C). The magnitude of reconstructed elevations of  $Ca_i$  measured using Oregon green-5N after dim flashes and their timing relative to that of the receptor potential were similar to those recorded from cells bathed in ASW. Similar results were obtained from a total of four cells. The correlation between the latencies of the receptor potential and the elevation of  $Ca_i$  cannot, therefore, be ascribed to the entrance of  $Ca^{2+}$  ions through light- or voltage-activated channels in the plasma membrane.

#### *Repeated Illumination in 0 Ca-ASW Increases the Latency and Slows the Rise Time of both the Elevation of $Ca_i$ and the Receptor Potential*

Bolsover and Brown (1985) demonstrated that repeated stimulation with bright flashes, while bathing cells in 0

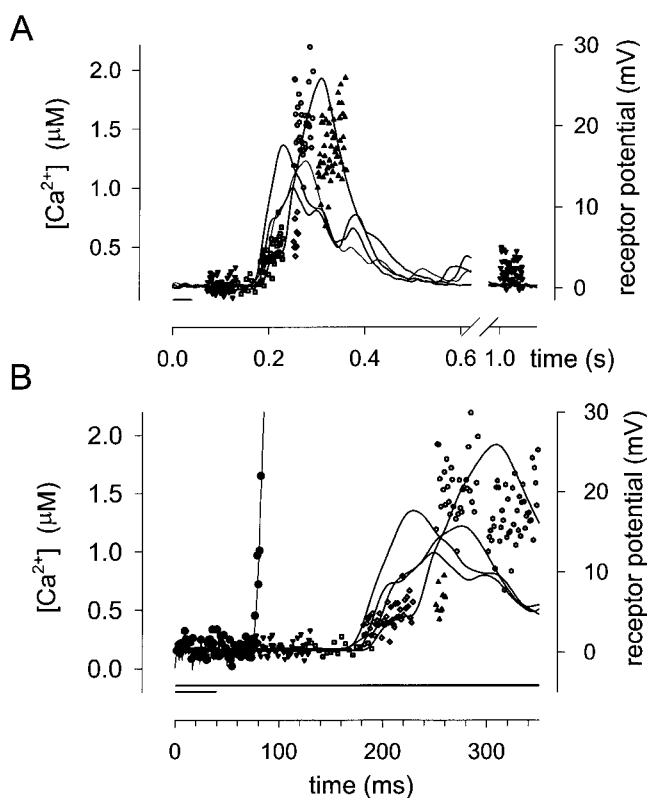


Figure 10. (A) Representative receptor potentials (solid lines) and reconstructed elevation of  $Ca_i$  (symbols) recorded from a chemically bleached photoreceptor filled with fluo-4 after a flash that delivered  $\sim 10$  effective photons. The bar beneath the traces indicates the timing of the flash, 40-ms duration,  $3 \log_{10} U$  attenuation. (B) The rising edge of the responses are shown on an expanded time scale, together with an estimate of  $Ca_i$  and a receptor potential recorded during a step of illumination by the unattenuated laser beam, delivering  $\sim 10^5$  effective photons/s. The bars below the traces indicate the onset and duration of the stimuli. Note the large fluctuations in the amplitude and latency of the representative receptor potentials after the dim flash.

Ca-ASW, prolongs the latency of the electrical response, slows, and diminishes it. Light-induced elevations of  $Ca_i$  estimated from recordings using aequorin are diminished under these conditions, indicating that the calcium stores become depleted by the repetitive illumination. We wished to determine whether the prolongation of the latency of the electrical response under these conditions was accompanied by a similar prolongation of the latency of the elevation of  $Ca_i$ . Bleached photoreceptors were bathed in 0 Ca-ASW and subjected to repetitive x-y scans by the unattenuated laser beam so as to uniformly deplete  $Ca^{2+}$  stores within the cell. At intervals between the scans, the elevation of  $Ca_i$  and receptor potential elicited by spot illumination at the edge of the R-lobe were measured. As the length of time in 0 Ca-ASW and the number of scans delivered increased, the latency of the elevation of  $Ca_i$  increased and its magnitude and rate of rise decreased (Fig. 12). Despite the large



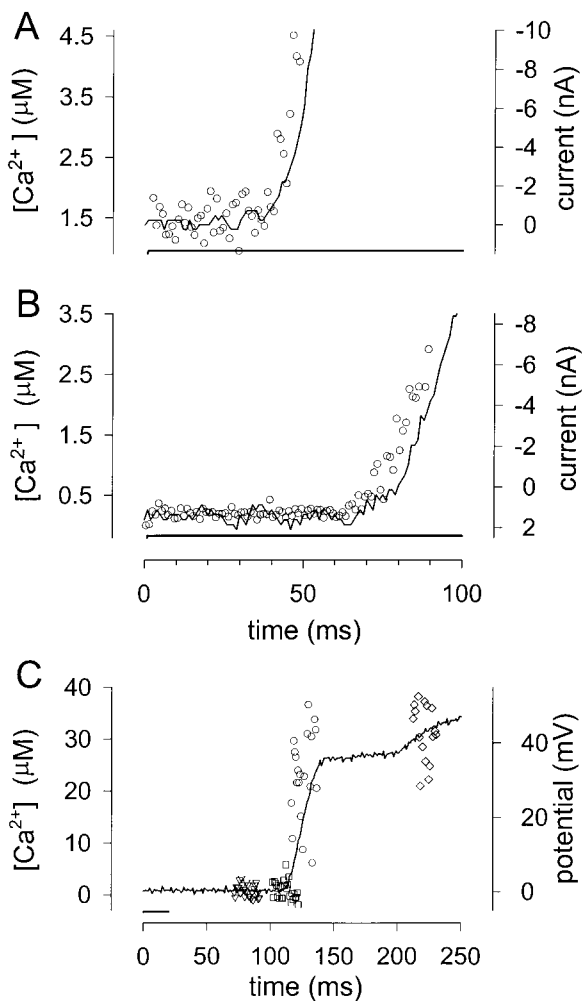


Figure 11. (A and B) Membrane current (solid lines; inward current is shown as an upward deflection) and estimated elevation of  $Ca_i$  ( $\circ$ ) recorded from a chemically bleached photoreceptor filled with fluo-4 that was voltage clamped to its resting potential ( $-55$  mV) during steps of illumination by the unattenuated laser beam, delivering  $\sim 10^5$  effective photons/s (bars below traces), recorded when the cell was bathed in ASW (A) and 15 min after switching to 0 Ca-ASW (B). (C) Representative receptor potential (solid line) and reconstructed elevation of  $Ca_i$  (symbols) recorded from a photoreceptor filled with Oregon green-5N that was bathed for 15 min in 0 Ca-ASW after a flash that delivered  $\sim 200$  effective photons. The flash (bar beneath trace), 20-ms duration,  $4 \log_{10} U$  attenuation, was delivered at the origin of the time axis.

differences in the absolute latency of the responses, the rise of the receptor potential was still approximately coincident with that of the elevation of  $Ca_i$ . Results similar to those of Fig. 12 were obtained in three other cells.

#### *The Elevation of $Ca_i$ Is Necessary for Generating a Rapid, Highly Amplified Electrical Response*

Bathing photoreceptors filled with fluo-4 in 0 Ca-ASW containing 100  $\mu M$  CPA, an inhibitor of endoplasmic reticulum (ER)  $Ca^{2+}$  ATPase (Mason et al., 1991; Walz et al., 1995), dramatically reduced the light-induced eleva-

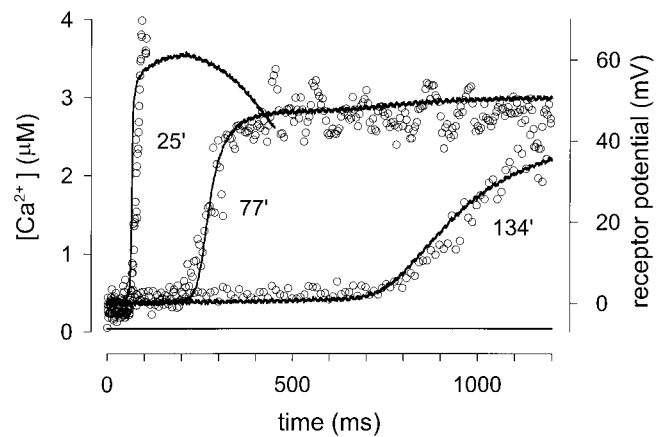


Figure 12. Receptor potentials (solid lines) and elevations of  $Ca_i$  ( $\circ$ ) recorded from a chemically bleached photoreceptor filled with fluo-4 during steps of illumination by the unattenuated laser beam (bar beneath traces), beginning at the origin of the time axis and delivering  $\sim 10^5$  effective photons/s, recorded (from the left) 25, 77, and 134 min after beginning bathing the cell in 0 Ca-ASW. Between each of these spot-mode measurements of an elevation of  $Ca_i$ , the cell was scanned  $256 \times$  in the x-y scan mode by the unattenuated laser so as to uniformly excite the photoreceptor and deplete calcium stores.

tion of  $Ca_i$  and slowed and diminished the receptor potential (Fig. 13 A). As reported by Dörlöchter et al. (1999), single photon signals were also reduced in amplitude to below the noise level (Fig. 13, B and C). However, despite the large reduction in the sensitivity of the cell to dim flashes, the receptor potential was never completely abolished by treatment with CPA in 0 Ca-ASW. If the response remaining after CPA treatment is considered to be a separate component of the transduction process, not dependent on  $Ca^{2+}$  release (Nagy, 1993), it is relatively insignificant during the rising edge of the normal response to a dim flash. For the cell of Fig. 13 A, the response after CPA treatment was 1.2 mV at 123 ms after the flash, compared with 25 mV at the same time before CPA treatment. However, as previously reported (Ukhanov and Payne, 1995), intense, saturating illumination by the unattenuated laser resulted in the generation of large receptor potentials in the absence of a significant elevation of  $Ca_i$  (Fig. 13 D). Thus, for bright flashes, the current generated by this mechanism may be significant. To check that the loss of the light-induced elevation of  $Ca_i$  was not caused by the loss of the  $Ca^{2+}$  indicator dye from the cytosol, the cell was bathed for 2 min in 10 mM Ca-ASW, without added CPA. Upon illumination of the chosen spot by the unattenuated laser beam, fluorescence rose after a latency of 30 ms, with a half time of 140 ms, to a peak that was  $4.0 \times$  its value in 0 Ca-ASW. This slower, but substantial, rise in fluorescence presumably reflects  $Ca^{2+}$  influx, as well as any renewed release from stores, and it demonstrates the presence of calcium indicator after the prolonged incubation with CPA. Results similar to those of Fig. 13 were obtained in a total of four cells.

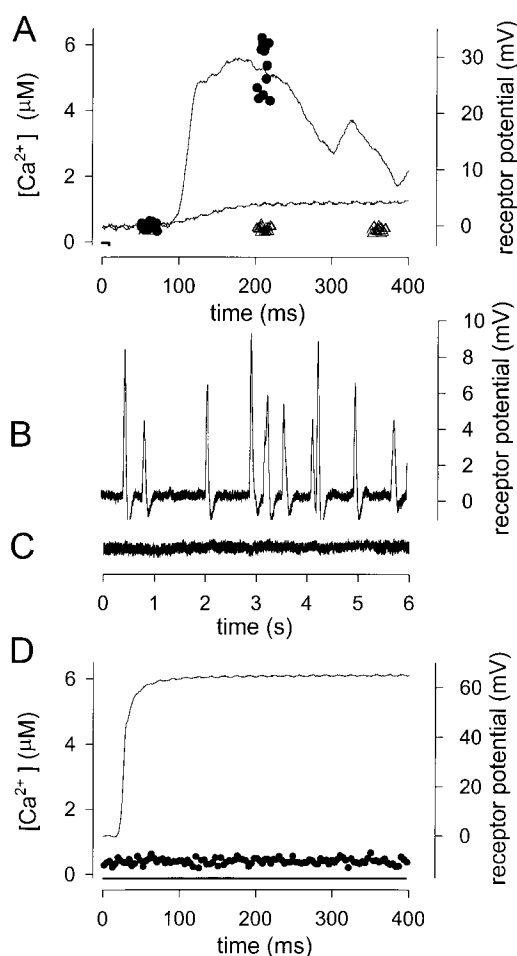


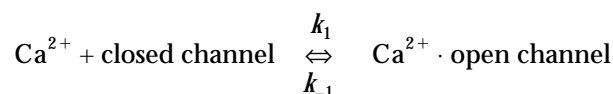
Figure 13. (A) Representative receptor potentials (solid lines) and elevations of  $\text{Ca}_i$  (symbols) recorded from a photoreceptor filled with fluo-4 after a flash that delivered  $\sim 100$  effective photons, when the cell was bathed for 10 min in 0 Ca-ASW (●) and 30 min after switching the bathing solution to 0 Ca-ASW containing 100  $\mu\text{M}$  cyclopiazonic acid (CPA; △). 5 min before the measurement in CPA, the cell was x-y scanned with the unattenuated laser beam so as to verify that the confocal spot was at the edge of the R-lobe, and to ensure depletion of  $\text{Ca}^{2+}$  stores. The flash (bar beneath traces), 10-ms duration,  $4 \log_{10}$  U attenuation, was delivered at the origin of the time axis. (B) Single photon signals (bumps), recorded during dim continuous illumination, when the cell was bathed for 10 min in 0 Ca-ASW, and (C) 30 min after switching the bathing solution to 0 Ca-ASW containing 100  $\mu\text{M}$  CPA. (D) Receptor potential (solid line) and estimated  $\text{Ca}_i$  (●) recorded from a chemically bleached photoreceptor during a step of illumination by the unattenuated laser beam (bar beneath trace), beginning at the origin of the time axis and delivering  $\sim 10^8$  effective photons/s, recorded 45 min after switching the bathing solution to 0 Ca-ASW containing 100  $\mu\text{M}$  CPA.

### Relationship between $\text{Ca}_i$ and Inward Current

The above result, our failure to ascribe the elevation of  $\text{Ca}_i$  to influx of  $\text{Ca}^{2+}$ , and the conclusions of previous work (see discussion) are consistent with the idea that the correlation between the timing of the light-induced

elevation of  $\text{Ca}_i$  and inward current might result from the activation of an inward current by released  $\text{Ca}^{2+}$ . As noted above, variation in the timing of the  $\text{Ca}^{2+}$  signal from cell to cell makes it impossible to define an accurate relationship between the estimated value of  $\text{Ca}_i$  and the receptor potential or the light-induced current. However, we thought it valuable to determine the approximate form of a process that might couple elevation of  $\text{Ca}_i$  to the receptor potential.

Fig. 14 shows the mean elevation of  $\text{Ca}_i$  and inward current recorded from five bleached photoreceptors filled with fluo-4 that were voltage clamped to their resting membrane potentials and illuminated with a step of light from the unattenuated laser. For each cell, the time taken for the light-induced current to reach a criterion of  $-2$  nA has been subtracted from the time base so as to compare the relative timing of  $\text{Ca}^{2+}$  and electrical signals, despite the variation in response latency. As with the voltage responses of Fig. 9, the elevation of  $\text{Ca}_i$  was detectable at the earliest times of the electrical response to light. For three of the five cells individually, as well as for the mean data in Fig. 9, elevation of  $\text{Ca}_i$  is detectable before significant inward current flow (detection based on a criterion of two successive samples  $>2$  SD above the initial noise level). To relate the elevation of  $\text{Ca}_i$  to inward current flow, a simple model was assumed in which we ignore the small component that is not dependent on  $\text{Ca}^{2+}$  release during the rising edge of the response and assume that  $\text{Ca}^{2+}$  reversibly binds to the light-activated channel or an associated protein. The channel was assumed to be open for as long as  $\text{Ca}^{2+}$  is bound.



For low  $[\text{Ca}^{2+}]$ , before saturation of the dye or inward current occurs, the rate of change of the mean inward current,  $i_L(t)$ , can be described by the differential equation:

$$di_L(t)/dt = k_1[\text{Ca}^{2+}](t) - k_{-1}i_L(t). \quad (6)$$

Fig. 14 shows a fit of Eq. 6 to  $i_L(t)$  obtained through numerical integration using the observed light-induced elevation of  $\text{Ca}_i$ ,  $k_1 = 0.35 \text{ nA} \cdot \mu\text{M}^{-1} \cdot \text{ms}^{-1}$  and  $k_{-1} = 0.2 \text{ ms}^{-1}$ . For constant  $[\text{Ca}^{2+}]$ , Eq. 6 then describes a first-order process with a sensitivity of inward current to  $\text{Ca}_i$  of  $1.75 \text{ nA} \cdot \mu\text{M}^{-1}$  and a time constant of 5 ms. Aside from parsimony, our choice of a linear relationship between  $[\text{Ca}^{2+}]$  and  $i_L$  is dictated by the following consideration. After the latent period, the rise of  $[\text{Ca}^{2+}]$  with time during the light step of Fig. 14 approximates to a linear ramp. Eq. 6 then predicts that the current will also rise as a linear ramp after a brief

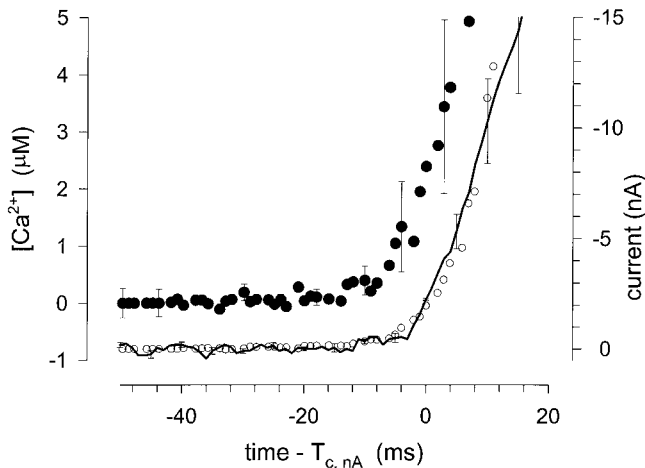


Figure 14. Mean elevation of  $\text{Ca}_i$  (●) and inward current (line) recorded from five photoreceptors filled with fluo-4 during successive steps of illumination delivered from the unattenuated laser beam. Error bars indicate SEM. To compensate for the variable latency of each response, the time,  $T_{c, \text{nA}}$ , taken for the inward current to exceed  $-2 \text{ nA}$  was determined for each response and was subtracted from the time base of each response. (○) Inward current predicted by Eq. 6, as described in the text.

delay, as is observed. Models that invoke a cooperative action of several calcium ions to activate the channel would predict a sigmoidal rise in current with time.

## DISCUSSION

Pharmacological experiments have indicated that light-induced release of  $\text{Ca}^{2+}$  is necessary, in *Limulus* photoreceptors, for a rapid, highly amplified initial response to dim flashes of light (Bolsover and Brown, 1985; Payne and Fein, 1986; Frank and Fein, 1991; Shin et al., 1993; Nagy and Contzen, 1997; Dörlöchter et al., 1999). Ours is the first measurement of an elevation of  $\text{Ca}_i$  that is sufficiently rapid to mediate this effect. Our reconstructed elevations of  $\text{Ca}_i$  are similar in peak amplitude ( $10\text{--}100 \mu\text{M}$ ) and duration ( $1\text{--}3 \text{ s}$ ) to those recorded previously using other methods (Brown and Blinks, 1974; Brown et al., 1977; Levy and Fein, 1985; O'Day and Gray-Keller, 1989). However, the high temporal resolution of fluorescent indicators and the focal origin of the fluorescence within a few microns of the photoreceptive membrane (Ukhanov and Payne, 1995) allow more accurate measurement of elevations of  $\text{Ca}_i$  that might modulate the activity of the light-sensitive conductance in the plasma membrane.

For a wide range of flash intensities and conditions, the detection of the electrical response of dark-adapted *Limulus* ventral photoreceptors is, within a few milliseconds, coincident with that of the elevation of  $\text{Ca}_i$ . For responses of five chemically bleached photoreceptors to steps of light, where an uninterrupted  $\text{Ca}^{2+}$  signal could be recorded, the mean receptor potential was in-

significant at the time that the elevation of  $\text{Ca}_i$  was detected. Elevation of  $\text{Ca}_i$  was detected in three of another five cells before significant inward current flowed under voltage clamp. These elevations of  $\text{Ca}_i$  appeared to result from the release of  $\text{Ca}^{2+}$  ions from intracellular stores, since they were undiminished by removal of extracellular  $\text{Ca}^{2+}$  ions, but were abolished by exposure to CPA. Thus, light-induced  $\text{Ca}^{2+}$  release precedes the generation of most, if not all, of the electrical response.

## Implications for Models of Visual Transduction by *Limulus* Ventral Photoreceptors

Our observations necessitate a revision of a previous model of a role for  $\text{Ca}^{2+}$  release in phototransduction in *Limulus* photoreceptors. Payne and Fein (1986) observed that the rising edge of the photocurrent rose too abruptly to be plausibly modeled by a linear cascade of first-order reactions, as was first proposed by Borsellino et al., 1965. To explain the abrupt rise, they proposed that a gradual light-induced elevation of  $\text{Ca}_i$  accelerated the rate of three sequential steps in the proposed cascade of reactions that link photoisomerized rhodopsin to the activation of ion channels in the plasma membrane. Our measurements of  $\text{Ca}_i$  are not consistent with a gradual elevation. It is apparent from inspection of Figs. 4, 6, and 7 that the elevation of  $\text{Ca}_i$  shares the abrupt rising edge of the electrical response to light.  $\text{InsP}_3$ -mediated calcium release in other cells also displays abrupt release after a variable latent period, a behavior that has been attributed to an apparent threshold concentration of  $\text{InsP}_3$  required to activate the  $\text{InsP}_3$  receptor in the ER. (Marchant and Taylor, 1997).

The simplest explanation for the correlation between the elevation of  $\text{Ca}_i$  and the receptor potential is that light-induced elevation of  $\text{Ca}_i$  activates a component of the photocurrent (Shin et al., 1993). Our simple analysis shows that a first-order process with a steady state sensitivity of inward current to  $\text{Ca}_i$  of  $1.75 \text{ nA} \cdot \mu\text{M}^{-1}$  and a time constant of  $5 \text{ ms}$  would model the relationship between the initial mean elevation of  $\text{Ca}_i$  and mean inward current recorded from five cells. However, variation in the timing of the  $\text{Ca}^{2+}$  signal relative to that of the inward current from cell to cell, possibly due to experimental error, makes it impossible to say definitively that such a deterministic linkage exists. Variation in timing of the  $\text{Ca}^{2+}$  signal may be due to error in placing the confocal measurement spot over the microvillar membrane. Assuming a diffusion coefficient for  $\text{Ca}^{2+}$  of  $227 \mu\text{m}^2 \cdot \text{s}^{-1}$  (Allbritton et al., 1992), an error of only  $2 \mu\text{m}$  could result in a delay of several milliseconds in the detection of  $\text{Ca}^{2+}$  released from the ER, which lies within  $100 \text{ nm}$  of the microvillar membrane (Calman and Chamberlain, 1982; Payne et al., 1988).

In support of the proposal above, rapid  $\text{Ca}^{2+}$  release by flash photolysis of caged  $\text{InsP}_3$  can activate an inward

current in *Limulus* ventral photoreceptors within  $2.5 \pm 3.3$  ms of the detection of the  $\text{InsP}_3$ -induced elevation of  $\text{Ca}_i$  (Ukhanov and Payne, 1997). The molecular mechanism of this activation is unknown, as is the identity of the channels carrying the current. In addition, pharmacological blockade of light-induced  $\text{Ca}^{2+}$  release greatly slows and diminishes the initial, transient component of the response of dark-adapted *Limulus* ventral photoreceptors to dim and moderate flash intensities (Fig. 13; Bolsover and Brown, 1985; Payne and Fein, 1986; Frank and Fein, 1991; Shin et al., 1993; Nagy and Contzen, 1997; Dorlöchter et al., 1999). However, even though treatment with CPA to eliminate measured  $\text{Ca}^{2+}$  release has a dramatic effect on the amplitude and rate of rise of the response to dim light flashes, it does not eliminate the electrical response to bright light. Thus, a slower, less sensitive, pathway of transduction might also exist that activates the light-sensitive conductance in the absence of calcium release (Nagy, 1993).

Since treatment with CPA abolishes the large quantum bumps that comprise the response of the cell to very dim illumination (Dorlöchter et al., 1999),  $\text{Ca}^{2+}$  release appears to be a necessary step in the bump-generating mechanism. The large peak amplitude of the quantum bump, which averages 5 mV (0.5 nA in voltage clamp recordings; Yeandle and Spiegler, 1973), and requires the activation of hundreds of ion channels (Bacigalupo and Lisman, 1983), may then be the consequence of the amplification inherent in  $\text{InsP}_3$ -induced  $\text{Ca}^{2+}$  release. We calculate that, for dim flashes, at least 300  $\text{Ca}^{2+}$  ions are released per effective photon at the peak of the response.

#### *The Time to Release $\text{Ca}^{2+}$ May Limit the Temporal Accuracy of Visual Transduction*

Previous attempts to demonstrate an elevation of  $\text{Ca}_i$  that precedes or accompanies the rising edge of the electrical response using aequorin or Arsenazo III as an indicator were not successful (Brown and Blinks, 1974; Payne and Flores, 1992; Stieve and Benner, 1992) and it has been argued that light-induced calcium release is not rapid enough to contribute to the initial electrical response of invertebrate photoreceptors (Clapham, 1996; Sakakibara et al., 1998). While this may be true for the photoreceptors of *Drosophila*, which are capable of an electrical response within 6 ms of a light stimulus (Hardie 1995), it is clearly not true of *Limulus* ventral photoreceptors. Our findings might also be relevant for photoreceptors of other slow-moving species, such as the file clam (del Pilar Gomez and Nasi, 1998) and the marine mollusk, *Hermisenda* (Sakakibara et al., 1998), where intracellular injection of  $\text{InsP}_3$  has been also shown to induce rapid bursts of inward current or depolarization. However, the price of the amplification inherent in the  $\text{Ca}^{2+}$  release mechanism may be a longer,

more variable, latent period of the response in *Limulus* photoreceptors. We have demonstrated that the elevation of  $\text{Ca}_i$  displays variation from flash to flash, similar to that of the receptor potential. Variation in the latency of the photocurrent in response to dim flashes results in an inaccuracy in the coding of temporal information, a component of "transducer noise" (Lillywhite and Laughlin, 1979).

We thank Dr. Alain Dabdoub and Ms. Monika Deshpande for critical reading of the manuscript and Dr. Ian Mather of the Animal and Poultry Science Department, University of Maryland, for the use of the confocal microscope.

This work was supported by National Institutes of Health EY07743.

Submitted: 29 December 1999

Revised: 20 April 2000

Accepted: 21 April 2000

#### REFERENCES

- Acharya, J.K., K. Jalink, R.W. Hardy, V. Hartenstein, and C.S. Zuker. 1997.  $\text{InsP}_3$  receptor is essential for growth and differentiation but not for vision in *Drosophila*. *Neuron*. 18:881–887.
- Allbritton, N.L., T. Meyer, and L. Stryer. 1992. Range of messenger action of calcium ion and inositol 1,4,5-trisphosphate. *Science*. 258:1812–1815.
- Arnon, A., B. Cook, C. Montell, Z. Selinger, and B. Minke. 1997. Calmodulin regulation of calcium stores in phototransduction of *Drosophila*. *Science*. 275:1119–1121.
- Bacigalupo, J., and J.E. Lisman. 1983. Single-channel currents activated by light in *Limulus* ventral photoreceptors. *Nature*. 304: 268–270.
- Bayer, D.S., and R.B. Barlow, Jr. 1978. *Limulus* ventral eye. Physiological properties of photoreceptor cells in an organ culture medium. *J. Gen. Physiol.* 72:539–563.
- Berridge, M.J., and R.F. Irvine. 1989. Inositol phosphates and cell signalling. *Nature*. 341:197–205.
- Bolsover, S.R., and J.E. Brown. 1985. Calcium ion, an intracellular messenger of light adaptation, also participates in excitation of *Limulus* photoreceptors. *J. Physiol.* 364:381–393.
- Borsellino, A., M.G.F. Fuortes, and T.G. Smith. 1965. Visual responses in *Limulus*. *Cold Spring Harbor Symp. Quant. Biol.* 30:429–443.
- Brown, J.E., and J.R. Blinks. 1974. Changes in intracellular free calcium concentration during illumination of invertebrate photoreceptors. Detection with aequorin. *J. Gen. Physiol.* 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. *J. Physiol.* 267:299–320.
- Brown, J.E., L.J. Rubin, A.J. Ghalayini, A.P. Tarver, R.F. Irvine, M.J. Berridge, and R.E. Anderson. 1984. myo-Inositol polyphosphate may be a messenger for visual excitation in *Limulus* photoreceptors. *Nature*. 311:160–163.
- Calman, B.G., and S.C. Chamberlain. 1982. Distinct lobes of *Limulus* ventral photoreceptors. II. Structure and ultrastructure. *J. Gen. Physiol.* 80:839–862.
- Chyb, S., P. Raghu, and R.C. Hardie. 1999. Polyunsaturated fatty acids activate the *Drosophila* light-sensitive channels TRP and TRPL. *Nature*. 397:255–259.
- Clapham, D.E. 1996. TRP is cracked but is CRAC TRP? *Neuron*. 16: 1069–1072.

- Clark, A.W., R. Millecchia, and A. Mauro. 1969. The ventral photoreceptor cells of *Limulus*. I. The microanatomy. *J. Gen. Physiol.* 54: 289–309.
- del Pilar Gomez, M., and E. Nasi. 1998. Membrane current induced by protein kinase C activators in rhabdomeric photoreceptors: implications for visual excitation. *J. Neurosci.* 18:5253–5263.
- Dorlöchter, M., and H. Stieve. 1997. The *Limulus* ventral photoreceptor: light response and the role of calcium in a classic preparation. *Prog. Neurobiol.* 53:451–515.
- Dorlöchter, M., W.J. Yuan, and H. Stieve. 1999. Effects of calcium and cyclopiazonic acid on the photoresponse in the *Limulus* ventral photoreceptor. *Z. Naturforsch. C Biosci.* 54:446–455.
- Faddis, M.N., and J.E. Brown. 1992. Flash photolysis of caged compounds in *Limulus* ventral photoreceptors. *J. Gen. Physiol.* 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.
- Frank, T.M., and A. Fein. 1991. The role of the inositol phosphate cascade in visual excitation of invertebrate microvillar photoreceptors. *J. Gen. Physiol.* 97:697–723.
- Gray-Keller, M.P., and P.B. Detwiler. 1996.  $\text{Ca}^{2+}$  dependence of dark- and light-adapted flash responses in rod photoreceptors. *Neuron*. 17:323–331.
- Gryniewicz, G., M. Poenie, and R.Y. Tsien. 1985. A new generation of  $\text{Ca}^{2+}$  indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440–3450.
- Johnson, E.C., M.P. Gray-Keller, and P.M. O'Day. 1998. Rescue of excitation by inositol following  $\text{Li}^{+}$ -induced block in *Limulus* ventral photoreceptors. *Vis. Neurosci.* 15:105–112.
- Hardie, R.C. 1995. Photolysis of caged  $\text{Ca}^{2+}$  facilitates and inactivates, but does not directly excite light-sensitive channels in *Drosophila* photoreceptors. *J. Neurosci.* 15:889–902.
- Kass, L., J.L. Pelletier, G.H. Renninger, and R.B. Barlow, Jr. 1988. Efferent neurotransmission of circadian rhythms in *Limulus* lateral eye. II. Intracellular recordings in vitro. *J. Comp. Physiol. A Sens. Neural Behav. Physiol.* 164:95–105.
- Kraemer, P., R. Lederhofer, J. Schnakenberg, and H. Stieve. 1989. A simple explanation for the large and widely differing time exponent of the initial response of *Limulus*. *J. Gen. Physiol.* 94:1117–1120.
- Levy, S., and A. Fein. 1985. Relationship between light sensitivity and intracellular free Ca concentration in *Limulus* ventral photoreceptors. A quantitative study using Ca-selective microelectrodes. *J. Gen. Physiol.* 85:805–841.
- Lillywhite, P.G., and S.B. Laughlin. 1979. Transducer noise in a photoreceptor. *Nature*. 277:569–572.
- Lisman, J.E., and Y. Sheline. 1976. Analysis of the rhodopsin cycle in *Limulus* ventral photoreceptors using the early receptor potential. *J. Gen. Physiol.* 68:487–501.
- Marchant, J.S., and C.W. Taylor. 1997. Cooperative activation of  $\text{IP}_3$  receptors by sequential binding of  $\text{IP}_3$  and  $\text{Ca}^{2+}$  safeguards against spontaneous activity. *Curr. Biol.* 7:510–518.
- Martinez, J.M. II, and R. Srebro. 1976. Calcium and the control of discrete wave latency in the ventral photoreceptor of *Limulus*. *J. Physiol.* 261:535–562.
- Mason, M.J., C. Garcia-Rodriguez, and S. Grinstein. 1991. Coupling between intracellular  $\text{Ca}^{2+}$  stores and the  $\text{Ca}^{2+}$  permeability of the plasma membrane. Comparison of the effects of thapsigargin, 2,5-di-(tert-butyl)-1,4-hydroquinone, and cyclopiazonic acid in rat thymic lymphocytes. *J. Biol. Chem.* 266:20856–20862.
- Nagy, K. 1993. Cyclic nucleotides and inositol trisphosphate activate different components of the receptor current in *Limulus* ventral nerve photoreceptors. *Neurosci. Lett.* 152:1–4.
- Nagy, K., and K. Contzen. 1997. Inhibition of phospholipase C by U-73122 blocks one component of the receptor current in *Limulus* photoreceptor. *Vis. Neurosci.* 14:995–998.
- O'Day, P.M., and M.P. Gray-Keller. 1989. Evidence for electrogenic  $\text{Na}^{+}/\text{Ca}^{2+}$  exchange in *Limulus* ventral photoreceptors. *J. Gen. Physiol.* 93:473–494.
- Oberwinkler, J., and D.G. Stavenga. 1998. Light dependence of calcium and membrane potential measured in blowfly photoreceptors in vivo. *J. Gen. Physiol.* 112:113–124.
- Payne, R., D.W. Corson, and A. Fein. 1986. Pressure injection of calcium both excites and adapts *Limulus* ventral photoreceptors. *J. Gen. Physiol.* 88:107–126.
- Payne, R., and A. Fein. 1986. The initial response of *Limulus* ventral photoreceptors to bright flashes. Released calcium as a synergist to excitation. *J. Gen. Physiol.* 87:243–269.
- Payne, R., and T.M. Flores. 1992. The latency of the response of *Limulus* photoreceptors to inositol trisphosphate lacks the calcium-sensitivity of that to light. *J. Comp. Physiol. A Sens. Neural Behav. Physiol.* 170:311–316.
- 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. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 320:359–379.
- Peretz, A., E. Suss-Toby, A. Rom-Glas, A. Arnon, R. Payne, and B. Minke. 1994. The light response of *Drosophila* photoreceptors is accompanied by an increase in cellular calcium: effects of specific mutations. *Neuron*. 12:1257–1267.
- Ranganathan, R., B.J. Bacskaï, R.Y. Tsien, and C.S. Zuker. 1994. Cytosolic calcium transients: spatial localization and role in *Drosophila* photoreceptor cell function. *Neuron*. 13:837–848.
- Ranganathan, R., D.M. Malicki, and C.S. Zuker. 1995. Signal transduction in *Drosophila* photoreceptors. *Annu. Rev. Neurosci.* 18: 283–317.
- Sakakibara, M., H. Inoue, and T. Yoshioka. 1998. Evidence for the involvement of inositol trisphosphate but not cyclic nucleotides in visual transduction in Hermissenda eye. *J. Biol. Chem.* 273: 20795–20801.
- Shin, J., E.A. Richard, and J.E. Lisman. 1993.  $\text{Ca}^{2+}$  is an obligatory intermediate in the excitation cascade of *Limulus* photoreceptors. *Neuron*. 11:845–855.
- Stieve, H., and S. Benner. 1992. The light-induced rise in cytosolic calcium starts later than the receptor current of the *Limulus* ventral photoreceptor. *Vision Res.* 32:403–416.
- Ukhanov, K., and R. Payne. 1995. Light activated calcium release in *Limulus* ventral photoreceptors as revealed by laser confocal microscopy. *Cell Calc.* 18:301–313.
- Ukhanov, K., and R. Payne. 1997. Rapid coupling of calcium release to depolarization in *Limulus* polyphemus ventral photoreceptors as revealed by microphotolysis and confocal microscopy. *J. Neurosci.* 17:1701–1709.
- Ukhanov, K.Y., T.M. Flores, H.S. Hsiao, P. Mohapatra, C.H. Pitts, and R. Payne. 1995. Measurement of cytosolic  $\text{Ca}^{2+}$  concentration in *Limulus* ventral photoreceptors using fluorescent dyes. *J. Gen. Physiol.* 105:95–116.
- Walz, B., O. Baumann, B. Zimmermann, and E.V. Ciriacy-Wantrup. 1995. Caffeine- and ryanodine-sensitive  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release from the endoplasmic reticulum in honeybee photoreceptors. *J. Gen. Physiol.* 105:537–567.
- Yeandle, S., and J.B. Spiegler. 1973. Light-evoked and spontaneous discrete waves in the ventral nerve photoreceptor of *Limulus*. *J. Gen. Physiol.* 61:552–571.
- Yeandle, S., and R. Srebro. 1970. Latency fluctuations of discrete waves in the *Limulus* photoreceptor. *J. Opt. Soc. Am.* 60:398–401.