

Inositol 1,4,5 Trisphosphate Releases Calcium from Specialized Sites within *Limulus* Photoreceptors

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Abstract. We have investigated the subcellular distribution and identity of inositol trisphosphate (InsP₃)-sensitive calcium stores in living *Limulus* ventral photoreceptor cells, where light and InsP₃ are known to raise intracellular calcium. We injected ventral photoreceptor cells with the photoprotein aequorin and viewed its luminescence with an image intensifier. InsP₃ only elicited detectable aequorin luminescence when injected into the light-sensitive rhabdomeral (R)-lobe where aequorin luminescence induced by light was also confined. Calcium stores released by

light and InsP₃ are therefore localized to the R-lobe. Within the R-lobe, InsP₃-induced aequorin luminescence was further confined around the injection site, due to rapid dilution and/or degradation of injected InsP₃. Prominent cisternae of smooth endoplasmic reticulum are uniquely localized within the cell beneath the microvillar surface of the R-lobe (Calman, B., and S. Chamberlain, 1982, *J. Gen. Physiol.*, 80:839-862). These cisternae are the probable site of InsP₃ action.

THE intracellular messenger, inositol 1,4,5 trisphosphate (InsP₃)¹, is the product of agonist-induced hydrolysis of phosphatidylinositol 4,5 bisphosphate, a minor component of the phospholipid in the plasma membrane (1, 2, 12, 25). InsP₃ fulfills its role as a messenger by releasing calcium from endoplasmic reticulum (ER) (20, 23, 24). For InsP₃ to function efficiently as an intracellular messenger, one might expect the InsP₃-sensitive ER to be located close both to the areas of plasma membrane where hydrolysis of phosphatidylinositol 4,5 bisphosphate occurs and to the sites of action of the released calcium. However, the identity and distribution of InsP₃-sensitive ER and the extent to which Ca-release by InsP₃ is localized within cells are unknown.

The photoreceptor cells of the ventral eye of the horseshoe crab *Limulus polyphemus* (16) are highly suitable for studying the subcellular distribution of InsP₃-induced calcium release. The photoreceptors are divided into light-sensitive rhabdomeral (R) lobes and light-insensitive arhabdomeral (A) lobes (7, 22). Injection of InsP₃ into the photoreceptors mimics the effect of light (4, 11) insofar as it depolarizes them (excitation) and then induces desensitization to subsequent light flashes (adaptation). InsP₃ has therefore been proposed as a second messenger in invertebrate visual transduction (4, 11).

Both illumination of ventral photoreceptors and the intracellular injection of InsP₃ release calcium from internal stores (5, 6, 17). The resultant rise in intracellular free calcium (Ca_i) can be detected using the luminescent pho-

toprotein aequorin (5, 21). The calcium released by InsP₃ causes both excitation (17) and adaptation of the photoreceptor (15, 17). Moreover, the effect of injection of calcium or InsP₃ is localized within the cell. Pressure injection of calcium into the R-lobe, but not the A-lobe, produces a transient depolarization of the cell and a desensitization of subsequent responses to light (19). InsP₃ is also more effective in depolarizing and adapting the cell when injected into the R- rather than the A-lobe (4, 11). There are two possible explanations for these findings. Either InsP₃ releases calcium from both the R- and the A-lobe, but only calcium released in the R-lobe is able to excite and adapt the cell, or InsP₃ is only able to release calcium in the R-lobe. We have used aequorin to detect transient elevations of Ca_i caused by localized injections of calcium and InsP₃ and we find that the second explanation is correct. The R-lobe therefore contains stores of calcium sensitive to release by InsP₃ that are not present in the A-lobe.

We suggest that InsP₃ specifically releases calcium from cisternae of smooth ER that underly the photoreceptive plasma membrane (7, 26, 27) and that are uniquely localized to the R-lobe (7). It is possible that other cell types have a nonuniform subcellular distribution of InsP₃-sensitive ER and this may be a general mechanism used to locally release calcium at its site of action.

Materials and Methods

Recording and Stimulation

Conventional methods of recording and stimulation, described in detail elsewhere (9, 10, 16), were used for intracellular recording from ventral nerve photoreceptors of *Limulus polyphemus*. Cells were stripped of glia in

1. *Abbreviations used in this paper:* A, arhabdomeral; Ca_i, intracellular free calcium ion concentration; ER, endoplasmic reticulum, InsP₃, inositol 1,4,5 trisphosphate; R, rhabdomeral.

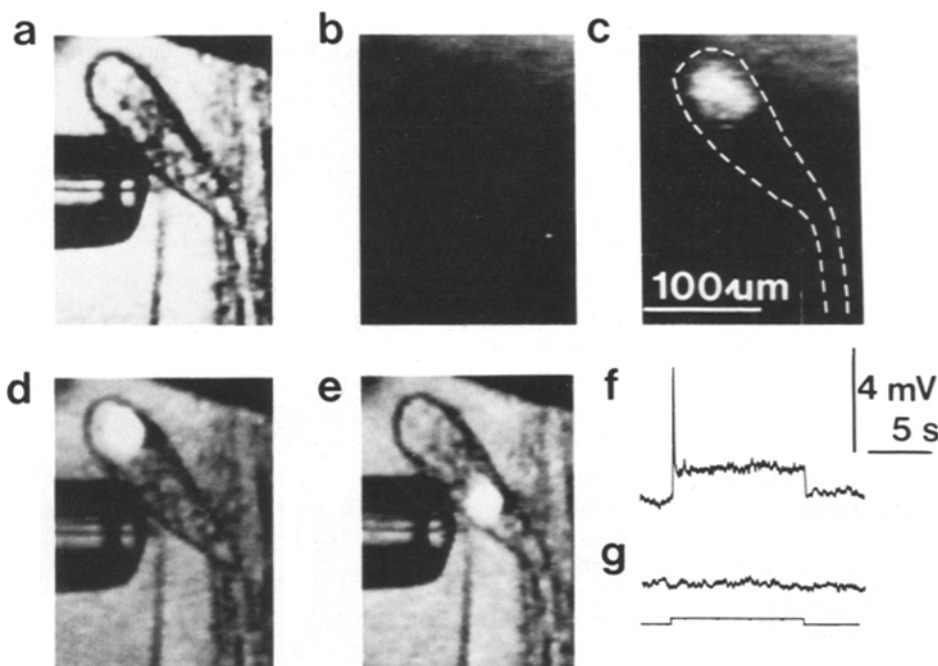


Figure 1. Localization of light-induced aequorin luminescence. (a) Video picture, taken using infrared illumination, of a cell stripped of glia and held away from the nerve by a suction pipette. The R-lobe is the region of the cell distal from the axon. A micropipette filled with aequorin, dissolved in carrier solution, impales the cell at its midpoint, to the right of the suction pipette. (b) Photograph of the photoreceptor in darkness after intracellular injection of 10–100 pl of aequorin solution, showing the level of background activity within the image intensifier. (c) Aequorin luminescence within the photoreceptor after the delivery of a 10-ms white light flash that uniformly illuminated the entire field of view and that saturated the receptor potential. (d–g) Scan of sensitivity of the photoreceptor to light. A spot of light illuminated the R-lobe (d) and A-lobe (e), eliciting a depolarization from the R-lobe (f) but not from the A-lobe (g).

some experiments so as to expose the R- and A-lobes (22). In other experiments, where it was not practical to strip the glia from the cell, the cell was scanned by light from a spot 10 μm in diameter after impalement (18) to determine the proximity of the intracellular micropipettes to the light-sensitive area of the cell. Cells were stimulated by white light from a 45 W tungsten-halogen lamp (Sylvania Industrial/Commercial Lighting, Danvers, MA), delivering 40 mW/cm^2 energy when focused onto the preparation.

Imaging of Aequorin Luminescence

Either of two long working length objectives (model UDI6X/NA0.17; Carl Zeiss Inc., New York, NY, for the experiment of Fig. 1 and model M-Plan 20X/NA0.4; Nikon Inc., Garden City, NY, for all other experiments) were used to image aequorin-loaded cells onto an image intensifier (Zeiss TV3M; Venus Scientific Inc., Farmingdale, NY). A shutter that opened 40 ms after the light flashes used to stimulate the photoreceptors prevented saturation of the intensifier by the stimulating light. The resulting video image was recorded on VHS tape (Maxell T-120HGX) for the experiment of Fig. 1 or 3/4-inch tape (Sony KCS-20BRK) in all other experiments. Relevant sections of the tape were stored on the video disk of a motion analyzer (Sony

model SVM-1010). 2-s sequences of the frames stored in the motion analyzer were displayed on a TV monitor and photographed by a camera loaded with Polaroid Type 667 film. The luminescence recorded on the video frames was temporally integrated by continuous exposure of the camera's film during each 2-s sequence. Aequorin luminescence elicited by light and injections of InsP_3 lasted 1–2 s (5, 17, 19) so that the photographs temporally integrated the entire recorded luminescence signal.

The following procedure was used to verify that the aequorin luminescence did not saturate the recorded video signal. A small photodiode (model 111A; United Detector Technology, Santa Monica, CA) was placed over the image on the TV monitor of the brightest aequorin luminescence recorded during the experimental series (the light-induced luminescence from the cell of Fig. 2). The peak intensity of the recorded luminescence was measured with the photodiode. A 60- μm spot of light was then focused onto the specimen plane (9, 10, 18) of the microscope and the image of the spot was recorded through the image intensifier onto videotape. The intensity of light delivered to the spot was increased in steps of 0.5 \log_{10} units from the threshold of detection to an intensity 2 \log units greater, using calibrated neutral density filters. The videotape of the spot was then played back to the TV monitor and the photodiode was placed over the recorded image of the spot. Over the range of intensities investigated, the relationship between

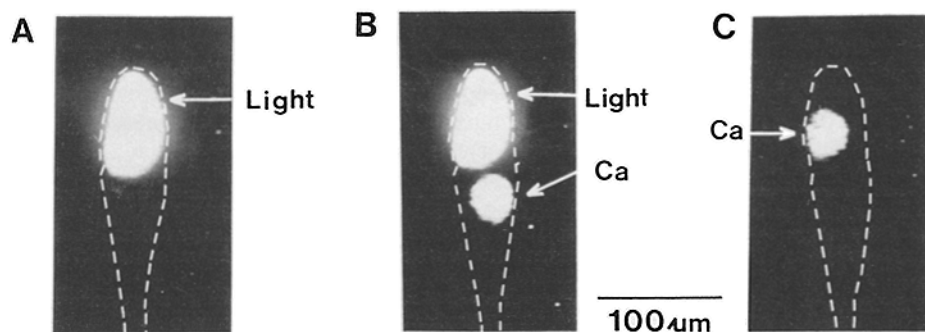


Figure 2. Aequorin luminescence elicited by light and by calcium injections. (A) Light-induced aequorin luminescence after the delivery of a 10-ms white light flash that uniformly illuminated the entire field of view. The dashed line outlines the cell body, which was left attached to the nerve. The point of departure of the axon from the A-lobe was clearly visible. (B) Aequorin luminescence elicited by light (upper luminescence) and by a 1–10-pl injection of 2

mM calcium chloride (pressure, 20 psi; duration, 100 ms) from a pipette that impaled the A-lobe of the photoreceptor (lower luminescence). The photograph is a superimposition of the light-induced luminescence recorded in A and the subsequent luminescence recorded during the injection of calcium. (C) Aequorin luminescence after a 1–10-pl injection of 2 mM calcium chloride (pressure, 20 psi; duration, 100 ms) into the R-lobe of the same cell as in A.

the intensity of the light delivered to the spot and the intensity of the recorded image of the spot showed no saturation. Since the intensity of the recorded image of the brightest light spot was twofold greater than the peak intensity of the recorded image of the brightest aequorin luminescence, the images of aequorin luminescence would appear to lie within the useful dynamic range of the camera and video recorder.

Pressure Injection of Substances into Cells

Rapid pressure injection of substances into the cells was achieved by applying brief (50–200 ms) pressure pulses to the back of a blunt micropipette that impaled the cell (8). In some experiments the cell was impaled with two such pipettes so as to inject either of two solutions. Isopotential electrical recordings of a cell's response to light gave unequivocal confirmation that the micropipettes impaled the same cell. Before the cell was impaled, ejections of solution were made into an oil droplet and the injected volume estimated. Single injections delivered ~1 pl into the oil drop. This volume was used to estimate volumes of 1–10 pl injected into the cell (8). Injection sites on the cells were unequivocally confirmed by viewing, under infrared illumination, disturbances in the cell's cytoplasm that were created by additional injections delivered after the injection for which aequorin luminescence was recorded (8).

The reliability of successive injections from the same pipette into a given cell was monitored by measuring the intensity of the recorded aequorin luminescence elicited by eight pairs of successive injections of 2 mM CaCl_2 into the A-lobe of four cells that had previously been loaded with aequorin (for example, Fig. 2 B). A photodiode (see above) was placed over the image of the aequorin luminescence on the TV monitor. The peak intensity of the recorded luminescence after the second injection of the pairs differed from that after the first injection by only $14 \pm 13\%$ ($n = 8$; range 2–48%). This small variation is consistent with the reliability of the recorded electrical response to successive injections of InsP_3 (4, 11, 17) and calcium (19). The large uncertainty (8) in the estimation of volumes injected into cells (1–10 pl) from volumes injected into oil (1 pl) would therefore appear to result from factors other than the variability of successive injection volumes from the same electrode.

Chemicals and Injection Solutions

The artificial seawater used in the experiment of Fig. 1 contained 435 mM NaCl, 10 mM KCl, 20 mM MgCl_2 , 25 mM MgSO_4 , 10 mM CaCl_2 , and 10 mM Hepes, pH 7.0. For the experiments of Figs. 2 and 3, involving multiple impalements of the cells by micropipettes, the calcium concentration of the artificial seawater was reduced to 1 mM by replacing CaCl_2 with NaCl, to reduce leakage of calcium into the cell and consequent desensitization to light and InsP_3 .

InsP_3 was the generous gift of Dr. C. E. Ballou and was prepared as described by Brockerhoff and Ballou (3). All injected chemicals were dissolved in carrier solution (100 mM potassium aspartate, 10 mM Hepes, pH 7.0) before loading into micropipettes. Aequorin was the generous gift of Dr. O. Shimomura (Marine Biological Laboratory, Woods Hole, MA). It was diluted to a final concentration of 7 mg/ml in carrier solution to which 100 μM EGTA was also added to prevent discharge of the aequorin before injection. 10–100 pl of aequorin solution was injected into the photoreceptors by a series of pressure pulses as described above.

Results

Localization of Light-induced Aequorin Luminescence

We first investigated the localization of light-induced calcium release within the cell's two lobes. The cell of Fig. 1 *a* was first injected with aequorin and dark adapted for several minutes. A flash of light that uniformly illuminated the entire photoreceptor elicited aequorin luminescence only in the region of the cell distal from the axon (Fig. 1 *b* and *c*). Illumination of the distal region with a spot of light (Fig. 1 *d*) elicited a depolarization (Fig. 1 *f*) that was not seen when the proximal region was similarly illuminated (Fig. 1 *e* and

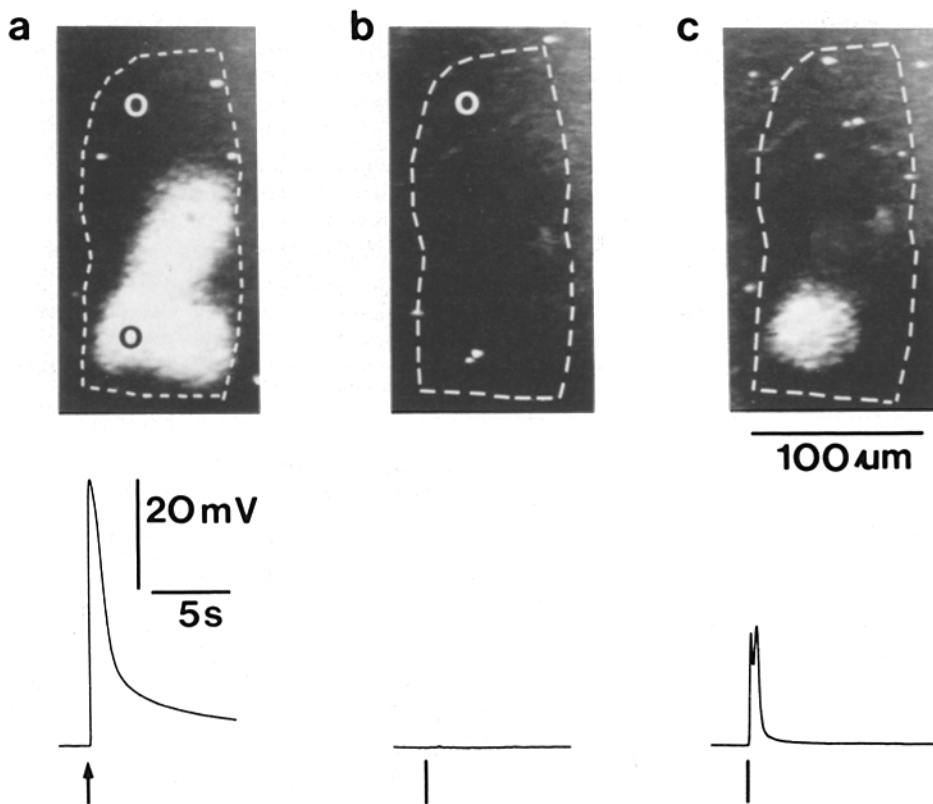


Figure 3. Aequorin luminescence elicited by light and by InsP_3 injections. (*a*) Aequorin luminescence induced in a ventral photoreceptor by a 10-ms flash of light that illuminated the entire field of view. The dashed line outlines the cell body, which was left attached to the nerve. Light-induced luminescence is localized within the cell to an area that defines the R-lobe. The area of the cell body where no luminescence occurred defines the A-lobe. The point of departure of the axon was not visible, probably because the axon departed from the underside of the A-lobe. The circles show sites of injection of InsP_3 into the A-lobe (white circle) and R-lobe (dark circle), as described below. The receptor potential is shown beneath the photograph. The arrow indicates the time of delivery of the flash. (*b*) Lack of aequorin luminescence after a 70-ms injection of 100 μM InsP_3 into the A-lobe. The InsP_3 was pressure injected (20 psi) from a micropipette containing 100 μM InsP_3 dissolved in carrier solution that impaled the cell at the white

circle. No electrical response followed the injection (*bottom trace*). (*c*) Aequorin luminescence elicited by a 70-ms injection of 100 μM InsP_3 from the same electrode as in *b* when the electrode impaled the R-lobe at the site of the black circle shown in *a*. The depolarization accompanying this injection is shown beneath the photograph.

g), unequivocally identifying these regions as the R- and A-lobes, respectively.

In experiments on more than 20 cells, aequorin luminescence was only detected in regions displaying the greatest sensitivity to depolarization by light. Occasionally, we observed two areas of aequorin luminescence at opposite ends of a cell. In these cases (four cells) we were always able to map two corresponding regions of light sensitivity, indicating the presence of two R-lobes (7, 22). The presence of two areas of luminescence indicates that aequorin diffuses across the A-lobe. We never found any dependence of luminescence on the proximity of the site of aequorin injection to the R-lobe.

Localization of Calcium-induced Aequorin Luminescence

We also investigated the aequorin luminescence after 1–10- μ l injections of 2 mM calcium chloride (Fig. 2). We impaled a cell with two micropipettes, one containing aequorin, the other containing 2 mM calcium chloride. The cells were loaded with aequorin from the first micropipette. We next recorded aequorin luminescence from the photoreceptor after a diffuse light flash that uniformly illuminated the cell, so as to define the area of light-induced aequorin luminescence, the R-lobe (Fig. 2 A). A subsequent 1–10- μ l injection of calcium from the other micropipette, which impaled the A-lobe, elicited localized aequorin luminescence within a region 30–50- μ m in diameter centered on the site of injection, but outside the area of light-induced aequorin luminescence (Fig. 2 B). This indicates that even though light induced no luminescence in the A-lobe, aequorin was present there. The injection of calcium was repeated two more times, producing a similar aequorin luminescence after each injection, to demonstrate the reliability of successive injections from the same electrode (see Materials and Methods).

To determine the relative efficiency of the aequorin in the A- and R-lobes, the micropipette containing calcium was withdrawn from the A-lobe and placed in the R-lobe. An injection of calcium was delivered into the R-lobe using the same pressure and duration of injection as for the injection into the A-lobe (Fig. 2 C). This injection elicited aequorin luminescence similar in intensity and area to that elicited by the injection into the A-lobe. Aequorin therefore diffuses throughout the cell and can indicate a rise in Ca_i with approximately equal efficiency in both lobes. Calcium appears to spread from the site of injection in both lobes over an area at least 30–50 μ m in diameter. Similar results were obtained in three other cells.

Localization of $InsP_3$ -induced Calcium Release

The cell of Fig. 3 was first loaded with aequorin and then penetrated in the A-lobe with a second micropipette that contained $InsP_3$. A brief, bright flash that uniformly illuminated the entire cell at the end of the experiment revealed the pattern of light-induced aequorin luminescence (Fig. 3 a) and so indicated the division of the cell body into A- and R-lobes. The depolarization of the cell by the light flash is also shown in Fig. 3 a. Before this flash, when the cell was dark adapted, injection of $InsP_3$ into the A-lobe, where light was unable to raise Ca_i , elicited neither a detectable aequorin luminescence (Fig. 3 b) nor a depolarization. Two con-

secutive injections of $InsP_3$ at this site induced three very small (1–2 mV) waves of depolarization after a delay of 3 s, probably due to diffusion of $InsP_3$ from the A-lobe to the R-lobe (4, 11), but no aequorin luminescence was detectable by our camera.

The micropipette containing $InsP_3$ was then withdrawn and reinserted into the R-lobe. The same pressure-pulse applied to the micropipette again injected $InsP_3$ into the dark-adapted cell, this time eliciting a rapid depolarization accompanied by a 40- μ m-diam aequorin luminescence, centered on the site of injection (Fig. 3 c). Comparison of a and c in Fig. 3 shows that the $InsP_3$ -induced aequorin luminescence is limited to a subregion of light-induced aequorin luminescence. This experiment was successfully completed in four cells, with similar results. In addition, nine cells were injected with $InsP_3$ in the A-lobe only, with no resulting aequorin luminescence being detected, although light elicited luminescence from the R-lobes of all of these cells. Injection of $InsP_3$ into the R-lobes of 14 other cells elicited luminescence in a subregion of the R-lobe, as in Fig. 3 c.

Discussion

The experiment of Fig. 2 shows that aequorin can signal a rise in Ca_i in both A- and R-lobes. We must therefore attribute the confinement of light- and $InsP_3$ -induced aequorin luminescence to the R-lobe as indicating a localized rise in Ca_i there. Localized light-induced calcium transients within ventral photoreceptors have been previously observed using the calcium-indicator dye, arsenazo III (13). Our study also confirms the greater amplitude of light-induced calcium transients recorded using calcium-sensitive electrodes when they impale light sensitive, rather than insensitive, regions of ventral photoreceptors (14). However, the latter study did reveal a small light-induced rise in Ca_i in the A-lobe, ~ 30 times less than that in the R-lobe, which appears to result from diffusion of calcium from the R- to the A-lobe. Our detection system, using aequorin and an image intensifier, is not sufficiently sensitive to reveal this elevation of Ca_i in the A-lobe. The peak light-induced rise in Ca_i in the A-lobe, as detected by Ca-sensitive electrodes, is ~ 1 μ M, while that in the R-lobe, which we can detect with our image intensifier, is ~ 30 μ M (14). Therefore our image intensifier would appear to be sensitive to increases in Ca_i of the order of 10 μ M.

The Identity and Distribution of the $InsP_3$ -sensitive Compartment

The localized ability of $InsP_3$ to release calcium within the cell is, to our knowledge, a novel finding. Fractionation studies of other cell types have implicated ER as the target of $InsP_3$ action (20, 23). Since ER is found throughout ventral photoreceptors (7), we suggest that not all ER within the cell is an $InsP_3$ -sensitive calcium store. The R-lobe, which contains the $InsP_3$ -sensitive calcium store, appears devoid of rough ER but it contains cisternae of smooth ER that accumulate calcium (26, 27) and are absent from the A-lobe. These cisternae of smooth ER are therefore the most likely candidate for the $InsP_3$ -sensitive calcium store.

In addition to the localization of the calcium stores to the R-lobe, the $InsP_3$ -induced release of calcium within the

R-lobe is further localized to a subregion around the injection site. This may explain why adaptation of the light response after injection of InsP_3 is also localized to the injection site (11).

We and others (see reference 14) have shown that the light-induced release of calcium is localized to the R-lobe. We propose that the cisternae of smooth ER are the light-sensitive store of calcium and that the release of calcium by light is mediated by light-induced production of InsP_3 (4). Elevation of Ca_i in the R-lobe, but not the A-lobe, is effective in exciting and adapting the cell (19), suggesting that the sites at which calcium is effective are also localized to the R-lobe. Thus the entire cascade, from the light-induced production of InsP_3 to the release of calcium and the response to calcium, appears to be localized to the R-lobe. The cisternae of smooth ER are ideally located to function as a calcium store since they lie within 0.1 μm of the microvilli that contain rhodopsin (7), where light-activated production of InsP_3 (4) might reasonably be expected to occur. Thus the R-lobe of ventral photoreceptors may exemplify the close proximity of sites of production of InsP_3 , InsP_3 -sensitive calcium stores, and calcium-sensitive receptors that is required for rapid and efficient intracellular signaling.

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