Neuron, Vol. 13, 837–848, October, 1994, Copyright © 1994 by Cell Press

Cytosolic Calcium Transients: Spatial Localization and Role in Drosophila Photoreceptor Cell Function

Rama Ranganathan,* Brian J. Bacskai,† Roger Y. Tsien,† and Charles S. Zuker*
*Departments of Biology and Neuroscience
†Department of Pharmacology
and Howard Hughes Medical Institute
University of California at San Diego
La Jolla, California 92093-0649

Summary

Drosophila phototransduction is a phosphoinositide-mediated and Ca^{2+}-regulated signaling cascade ideal for the dissection of feedback regulatory mechanisms. To study the roles of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in this process, we developed novel techniques for the measurement of $[Ca^{2+}]_i$ in intact photoreceptors. We genetically engineered flies that express a UV-specific rhodopsin in place of the normal rhodopsin, so that long wavelength light can be used to image $[Ca^{2+}]_i$ changes while minimally exciting the photoreceptor cells. We show that activation with UV generates $[Ca^{2+}]_i$ increases that are spatially localized to the rhabdomeres and that are entirely dependent on the influx of extracellular Ca^{2+}. Application of intracellular Ca^{2+} chelators of varying affinities demonstrates that the Ca^{2+} influx initially generates a large-amplitude transient that is crucial for negative regulation. Internal Ca^{2+} stores were revealed by discharging them with thapsigargin. But, in contrast to proposals that IP_3-sensitive stores mediate phototransduction, thapsigargin does not mimic or acutely interfere with photoexcitation. Finally, we identify a photoreceptor-specific PKC as essential for normal kinetics of $[Ca^{2+}]_i$ recovery.

Introduction

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) plays a crucial role as a second messenger in many signaling processes. Ca^{2+} has spatial and temporal dynamics more complex than any other known intracellular messenger (reviewed by Tsien and Tsien, 1990; Augustine and Neher, 1992a; Nowycky and Pinter, 1993). Ca^{2+} signals are initiated not by enzymatic synthesis (or breakdown), as for other messengers, but by ion channels that abruptly switch from closed to open states. Each open channel can admit millions of ions per second from the extracellular medium or from an organelle's lumen into the cytosol (Tsien and Tsien, 1990). Because very little Ca^{2+} is free in the cytosol of resting cells, the incoming flood of Ca^{2+} raises the concentration by a very large factor. In recent years, a number of studies have suggested that $[Ca^{2+}]_i$ gradients displaying specific intracellular compartmentalization may be the source of functionally relevant Ca^{2+} in modulating different cellular functions. The molecular mechanisms of Ca^{2+} action are very diverse and include the regulation of kinases and phosphatases to control protein phosphorylation, the formation of Ca^{2+}-calmodulin complexes that regulate the activity of many proteins, and direct binding to Ca^{2+}-sensitive cellular targets. In many cases, Ca^{2+} binding to specific regulatory sites triggers release of additional stored Ca^{2+}, leading to regenerative waves and oscillations of Ca^{2+}, which have been the subject of much recent interest (Meyer, 1991).

Ca^{2+} signals that occupy large fractions of a cell are easily studied with conventional imaging of fluorescent indicators and have been shown to participate in a wide variety of relatively slow and delocalized responses (see Tsien and Tsien, 1990, and references therein). On the other hand, transients within smaller subcellular volumes on the order of a few cubic microns are resolvable with the most advanced optical techniques and are believed to control cell responses in the subsecond to seconds range (Hernandez-Cruz et al., 1990). This includes fusion of large dense-cored vesicles in neurons and secretory cells (Thomas et al., 1990; Augustine and Neher, 1992a, 1992b), modulation of synaptic strength onto dendritic spines (Guthrie et al., 1991; Muller and Connor, 1991), chemotaxis (Brundage et al., 1993) and steering of growth cones and pseudopodia (Rehder and Kater, 1992; Zheng et al., 1994), and initiation of traveling waves in myocardium (Cheng et al., 1993). The very highest Ca^{2+} levels are reached in microseconds or milliseconds within tens or a hundred nanometers from the channel mouths. These domains are too small to be resolved directly by standard optical imaging of diffusely distributed indicators but have been postulated to control very fast responses, such as feedback on the channels themselves or exocytosis of primary neurotransmitters from small clear vesicles (Chad and Eckert, 1984; Fogelson and Zucker, 1985; Simon and Llinas, 1985).

An important requirement for the dissection of Ca^{2+} action in modulating complex signaling cascades would be the availability of a model system that allows a combination of molecular genetics, electrophysiology, and direct Ca^{2+} imaging. Drosophila melanogaster photoreceptor neurons are an ideal system for the study of $[Ca^{2+}]_i$ dynamics in signal transduction. First, phototransduction, the cellular process by which the energy of an absorbed photon is transduced into a graded change in the ionic permeabilities of the plasma membrane, has been well characterized genetically (Pak et al., 1970; Smith et al., 1991b; Zuker, 1992; Ranganathan et al., 1994). Second, excitation and regulation of this G protein-coupled signaling cascade can be monitored electrophysiologically with exquisite temporal resolution (millisecond kinetics; Hardie, 1991; Ranganathan et al., 1991). Third, $[Ca^{2+}]_i$ plays important regulatory roles in this cascade (reviewed by Ranganathan et al., 1994). Several important studies

utilizing fluorescent Ca^{2+} indicators, photoproteins, or Ca^{2+}-sensitive electrodes, have demonstrated light-induced changes in $[Ca^{2+}]_i$ in photoreceptors (Brown and Blinks, 1974; Brown et al., 1977; Nagy and Stieve, 1983; Payne and Fein, 1987; O'Day and Gray-Keller, 1989; Sandler and Kirschfeld, 1991; Levy and Payne, 1993; Walz et al., 1994). Of the above techniques, fluorescence imaging should potentially give the highest spatial and temporal resolution. Unfortunately, past attempts to measure light-induced current and fluorescence signals simultaneously in vertebrate (Ratto et al., 1988) and invertebrate (Peretz et al., 1994) photoreceptors have been compromised severely by the inability to separate functionally the stimulating light from that required for fluorescence excitation.

Phototransduction in Drosophila is mediated by a phospholipase C (PLC) effector molecule (Hotta and Benzer, 1970; Pak et al., 1970; Bloomquist et al., 1988); light-dependent activation of rhodopsin activates a heterotrimeric G protein of the G_q family (Lee et al., 1990) that regulates PLC activity (Scott, Hardy, and C. S. Z., unpublished data). PLC catalyzes the breakdown of the minor membrane phospholipid phosphotidyl inositol 4,5-bisphosphate (PIP2) into the well-known second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG; reviewed by Berridge, 1993). The actual signal that gates the light-activated membrane channels remains unknown, but proposed models have implicated either an IP_3-dependent elevation in $[Ca^{2+}]_i$ owing to release from IP_3-sensitive internal stores (Ca^{2+}-gating model; Minke and Selinger, 1991) or an IP_3-dependent depletion of internal pool Ca^{2+} (capacitative release model; Hardie and Minke, 1993). In the latter model, loss of Ca^{2+} from internal stores is proposed to mediate opening of plasma membrane channels through direct protein–protein communication between the membrane channels and an internal store Ca^{2+} sensor (Berridge, 1993).

Although the role of Ca^{2+} in excitation is not clear, we and others have shown previously that the light-activated channels are highly permeable to Ca^{2+} and that influx of extracellular Ca^{2+} ($[Ca^{2+}]_{out}$) is required for mediating sequential positive and negative feedback regulation of phototransduction (Hardie, 1991; Ranganathan et al., 1991). These regulatory circuits provide fundamental properties to photoreceptors: rapid response kinetics (Ranganathan et al., 1991) and broad dynamic response range (Hardie et al., 1993). The Ca^{2+}-dependent positive regulation is responsible for causing rapid onset of the light response and is mediated by as yet unknown mechanisms. The Ca^{2+}-dependent negative regulation is required for two related processes: rapid deactivation of the light response and adaptation (i.e., the mechanism that regulates the gain of photoexcitation to prevent response saturation). An electrophysiological screen for phototransduction mutants with abnormal light-activated current kinetics demonstrated that mutants in a photoreceptor cell–specific isoform of protein kinase C (eye-PKC; Schaeffer et al., 1989; Smith et al., 1991a) are specifically defective in all aspects of Ca^{2+}-dependent negative regulation (Ranganathan et al., 1991; Hardie et al., 1993). To study the spatial and temporal dynamics of $[Ca^{2+}]_i$ in this model G protein–coupled signaling cascade, we have now developed a preparation of Drosophila photoreceptors suitable for simultaneous high resolution Ca^{2+} imaging and electrophysiological analyses. We utilize this preparation to characterize the role of light-induced Ca^{2+} changes in intact photoreceptor cells.

Results and Discussion

Retuning Photoreceptor Spectral Sensitivity Allows Fluorescence Imaging of Ca^{2+}

Fluorescent indicators that reflect changes in Ca^{2+} levels as changes in fluorescence intensity or spectral properties have proven to be extremely useful in characterizing biological signal transduction processes (Tsien and Tsien, 1990). However, a fundamental problem that has prevented the use of these agents in the measurement of $[Ca^{2+}]_i$ in photoreceptors is the difficulty of using fluorescence techniques in cells that are themselves highly sensitive to light. For example, the extremely dense packing of receptor molecules in photoreceptor cells and the high photosensitive cross-sectional area of rhodopsin result in a quantum catch efficiency of 0.7–0.95 at the spectral absorption peak of rhodopsin, and dark-adapted cells generate saturating responses with less than 1% photoconversion of rhodopsin to the active state. In contrast, Ca^{2+}-indicator molecules typically have lower quantum efficiencies (0.05–0.5); only 1% or less of all the emitted photons are collected and detected, and thousands to millions of detection events are required for accurate measurements and high resolution images. These difficulties have prevented direct analyses of the kinetics of Ca^{2+} changes in response to activation and during deactivation of the phototransduction cascade.

To solve this problem, we reasoned that sufficient separation of the excitation spectra of rhodopsin and the Ca^{2+} indicator should allow us to measure indicator fluorescence with minimal cross-excitation of the photoreceptor cell. Figure 1A illustrates the action spectra of wild-type R1-6 Drosophila photoreceptors (Hardie, 1983; Feiler et al., 1988), showing that these cells have absorption spectra spanning the long-wavelength UV and visible spectra, a range that covers the excitation maxima of all currently available Ca^{2+} indicators (Tsien, 1989). To achieve acceptable spectral separation, we genetically retuned the spectral properties of R1-6 cells by generating transgenic animals expressing a modified rhodopsin in their R1-6 photoreceptor cells. These animals now express a UV-sensitive rhodopsin transgene (Rh1+4 λ_{max} = 365 nm; Feiler et al., 1992), in a genetic background carrying a deletion of the rhodopsin gene normally expressed in the R1-6 cells (*ninaE*^{17}; O'Tousa et al., 1985; Zuker et al., 1985). Figure 1B displays the action spec-

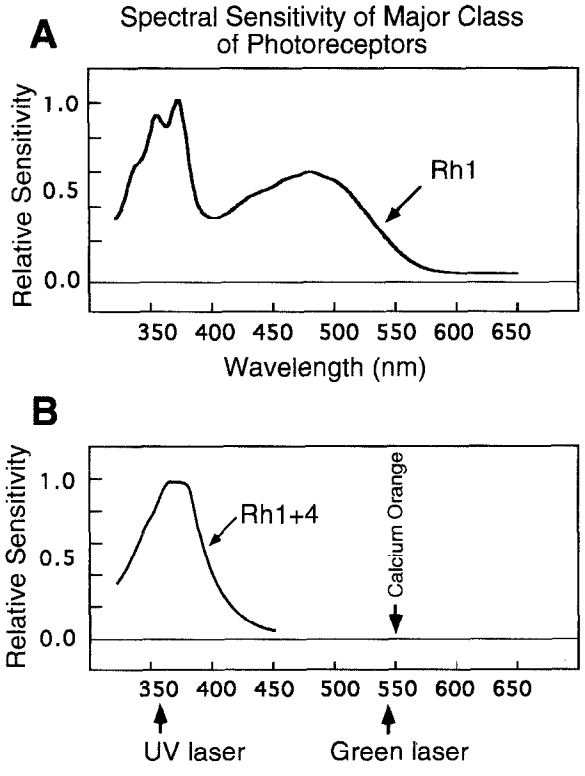


Figure 1. Spectral Properties of R1-6 Photoreceptors Expressing Rh1 versus Rh3 or Rh4

(A) Spectral sensitivity of R1-6 photoreceptors from wild-type flies expressing Rh1 rhodopsin (Feiler et al., 1988, 1992; Britt et al., 1993). The action spectra (from Feiler et al., 1992) were obtained from ERG measurements of white-eyed flies using the light-clamp technique of Franceschini (1979). The sensitivity of the cell is inversely related to light flux at any given wavelength. These cells are sensitive to UV as well as visible light, spanning the 350–600 nm range.

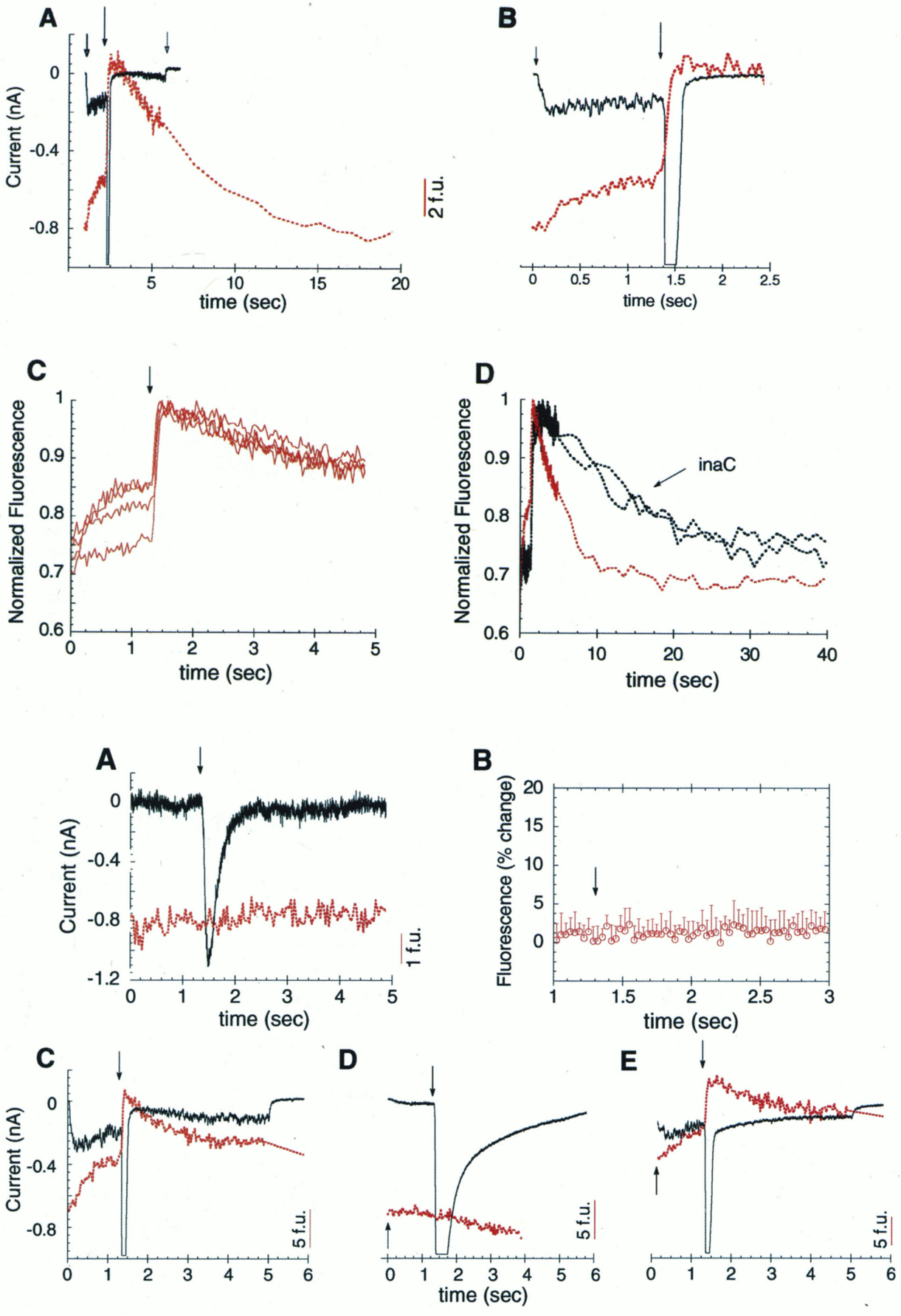
(B) Spectral sensitivity recordings from *ninaE;P[Rh1+4]* transgenic animals from Feiler et al., 1992 (see text for details). The UV light used to stimulate these cells was obtained from an argon laser providing 351 and 364 nm light. The green light used to excite the fluorescence Ca^{2+} indicator Ca-orange (Molecular Probes, Oregon) was obtained from a HeNe laser tuned to 543.5 nm. The Ca-orange dye has excitation maxima at 550 nm and emission maxima at approximately 575 nm (Molecular Probes).

tra of photoreceptors from *w;P[Rh1+4];ninaE*^{117} flies (referred to as Rh1+4), demonstrating the dramatic shift in their sensitivity, with responses dropping off steeply by 440 nm. These cells are otherwise physiologically similar to wild-type photoreceptor cells (Feiler et al., 1992). In the studies described below, we make use of these photoreceptors in combination with Calcium Orange (Ca-orange), a BAPTA-derived Ca^{2+} indicator with an excitation maximum at 554 nm and K_D = 328 nM (Molecular Probes, Oregon) to measure directly the dynamics of $[Ca^{2+}]_i$ during the light response.

Light-Dependent Changes in $[Ca^{2+}]_i$ Result from Extracellular Influx, not Internal Release

To analyze simultaneously, in real time, the light-dependent Ca^{2+} changes and the electrophysiological responses to light, we carried out whole-cell patch-clamp recordings in dissociated Rh1+4 photoreceptors internally dialyzed through the patch pipette with 200 μM Ca-orange. To achieve high temporal and spatial resolution in recording $[Ca^{2+}]_i$, the cells were imaged on a high speed laser scanning confocal microscope acquiring images at 30 frames per second (30 Hz sampling rate; see Experimental Procedures) (Tsien and Bacskai, 1994). All cells were first tested for threshold sensitivity (see Experimental Procedures) and allowed to dialyze for 5 min following establishment of whole-cell mode, to ensure equilibration of intracellular indicator concentrations. To compare directly the kinetics of $[Ca^{2+}]_i$ with the light-induced electrical response, we plotted the average fluorescence intensity of the cell together with light-activated currents (V_{hold} = −40 mV). Figures 2A and 2B show that strong pulses of 543.5 nm (green) light used to record Ca^{2+} levels evoke a small, noisy inward current in Rh1+4 photoreceptors and are associated with a slow Ca^{2+} rise that reaches a plateau typically within 1 s. These small, noisy responses are consistent with the extremely poor sensitivity of the photoreceptors at this wavelength (see Figure 1). In contrast, a superimposed weak UV flash (33 ms duration) results in a rapidly activating and deactivating electrical response associated with a rapid rise in $[Ca^{2+}]_i$. The kinetics of the UV-induced Ca^{2+} rise is similar to the rate of current activation (Figure 2B), consistent with the idea that a fraction of the light-activated current is carried by Ca^{2+} (Ranganathan et al., 1991; Hardie and Minke, 1992).

The light-dependent elevations of $[Ca^{2+}]_i$ could result from the release of Ca^{2+} from internal stores, entry of extracellular Ca^{2+}, or a combination of the two. To study the relative contributions of extracellular and internal pool Ca^{2+}, we recorded electrical responses and Ca^{2+} signals in Rh1+4 photoreceptors under varying concentrations of $[Ca^{2+}]_{out}$. Figure 3A shows that photoreceptors recorded in nominally zero $[Ca^{2+}]_{out}$ solutions show electrical responses with characteristic slow kinetics (Ranganathan et al., 1991) but show no associated changes in $[Ca^{2+}]_i$. Repeated attempts to improve the signal-to-noise ratio with signal averaging over individual acquisitions from multiple cells failed to reveal any Ca^{2+} transient in the absence of external Ca^{2+} (Figure 3B). To prove that these results are not due to artifactual depletion of internal stores resulting from incubation in bath solutions lacking Ca^{2+}, we recorded from photoreceptors in which we used rapid, local solution exchange to vary $[Ca^{2+}]_{out}$. Figures 3C–3E show that the normal rise in $[Ca^{2+}]_i$ in response to a UV flash with 1.5 mM $[Ca^{2+}]_{out}$ is instantly abolished by local perfusion with a zero Ca^{2+} solution but can be restored upon reintroduction of the high Ca^{2+} solution. Rather than an increase, under zero Ca^{2+} conditions, the cells show a light-dependent decrease in $[Ca^{2+}]_i$ (Figure 3D), possibly owing to efflux through the light-activated channels. Thus, although we cannot rule out the possibility that very small Ca^{2+}


A
Current (nA)
0
-0.4
-0.8
5
10
15
20
time (sec)
2 f.u.
B
0
0.5
1
1.5
2
2.5
time (sec)
C
Normalized Fluorescence
1
0.9
0.8
0.7
0.6
0
1
2
3
4
5
time (sec)
D
Normalized Fluorescence
inaC
0
10
20
30
40
time (sec)
A
Current (nA)
0
-0.4
-0.8
-1.2
1 f.u.
time (sec)
B
Fluorescence (% change)
20
15
10
5
0
1
1.5
2
2.5
3
time (sec)
C
Current (nA)
0
-0.4
-0.8
5 f.u.
D
5 f.u.
time (sec)
E
5 f.u.

changes below the threshold of detection may result from internal release (see below), influx of external Ca^{2+} is responsible for all measured light-dependent changes in $[Ca^{2+}]_i$.

Role of Internal Ca^{2+} Stores in Photoexcitation

The finding that extracellular Ca^{2+} is required for all measurable light-dependent changes in $[Ca^{2+}]_i$ is intriguing, since current models for photoexcitation in Drosophila propose an obligatory role for IP_3-mediated release of intracellular Ca^{2+} (Minke and Selinger, 1991; Hardie and Minke, 1993). To test these models, we attempted to demonstrate pharmacologically the presence of IP_3-sensitive internal Ca^{2+} pools in Drosophila photoreceptors by examining the effect of thapsigargin, a drug known to release Ca^{2+} from IP_3-sensitive stores. Since the molecular action of thapsigargin is to inhibit the re-uptake of Ca^{2+} into these internal stores and thereby cause irreversible store depletion (Thastrup et al., 1990), we should also be able to test the requirement of these stores for photoexcitation. Figure 4A shows that application of 1 μM thapsigargin to Rh1+4 photoreceptors results in a steady increase of $[Ca^{2+}]_i$ over several minutes, demonstrating that IP_3-sensitive stores in fact exist in these cells. Similar results were observed in the absence of external Ca^{2+} (data not shown), indicating that the source of Ca^{2+} is indeed intracellular. But thapsigargin application did not abolish the light response, even after complete depletion of stored Ca^{2+} as determined by full saturation of the rise in $[Ca^{2+}]_i$ (Figure 4A). Instead, the rise in $[Ca^{2+}]_i$ was associated only with a partial desensitization (adaptation) of the electrical response (Figure 4B), likely owing to activation of Ca^{2+}-dependent negative regulatory mechanisms (Brown, 1986; see below).

Since "capacitative" influx models in which the depletion of Ca^{2+} from IP_3-sensitive internal stores is transduced into opening of plasma membrane channels (Berridge, 1993; Putney and Bird, 1993) have also been proposed for mediating excitation (Hardie and Minke, 1993), we considered the possibility that thapsigargin-sensitive stores are involved in this process. If store depletion is the signal for excitation, pharmacological depletion of the stores should mimic light excitation. Figure 4B shows that depletion of internal Ca^{2+} by thapsigargin did not itself evoke any change in baseline conductance, indicating that these stores are unlikely to mediate photoexcitation through such a mechanism.

Average $[Ca^{2+}]_i$ Does Not Regulate Recovery from Adaptation

Since changes in $[Ca^{2+}]_i$ are known to drive feedback regulation of Drosophila phototransduction (reviewed by Ranganathan et al., 1994), it is possible that the dynamics of $[Ca^{2+}]_i$ determine the rate of these regulatory processes. To investigate this, we compared the kinetics of the light-dependent Ca^{2+} changes with the kinetics of photoreceptor adaptation. We measured recovery from adaptation as the recovery of sensitivity to 33 ms green test flashes that followed an adapting UV flash (Figure 5A, electrical trace). A comparison of the baseline conductance before and after the UV response shows that this response causes a rapid adaptation of the green light-induced current. Figure 5B shows that the kinetics of Ca^{2+} recovery is well correlated with the recovery from

Figure 2. Simultaneous Recording of Average $[Ca^{2+}]_i$ and Light-Activated Currents during a Light Response

In this and all following panels, electrical responses are shown in black, and $[Ca^{2+}]_i$ is shown in red. Scale for $[Ca^{2+}]_i$ is shown as a bar reporting arbitrary fluorescence units (F. U.).

(A) Recordings in Rh1+4 photoreceptors demonstrating that the kinetics of $[Ca^{2+}]_i$ rise follows the kinetics of photoreceptor cell activation. Cells were held at a potential of −40 mV and subjected to 5 s of continuous 543.5 nm (green) light to record $[Ca^{2+}]_i$ at maximal temporal resolution (30 Hz; this period is demarked by the two short arrows). The long arrow indicates the position of a 33 ms flash of UV light used to stimulate the photoreceptor cell. All recordings were done in physiological solutions with 1.5 mM $[Ca^{2+}]_{out}$ (see Experimental Procedures).

(B) The same response as in (A), with an expanded time scale to show details of the light-induced rise in $[Ca^{2+}]_i$. Note that the UV light causes adaptation of the maintained green response, consistent with Ca^{2+}-dependent desensitization (Ranganathan et al., 1994).

(C) A superimposition of light-induced $[Ca^{2+}]_i$ changes in four different cells. All Ca^{2+} responses were normalized to the peak response. The time of the UV flash is marked by the arrow.

(D) A comparison of the kinetics of light-induced $[Ca^{2+}]_i$ changes between Rh1+4 and mutants defective in a photoreceptor cell–specific PKC (inaC; Rh1+4) (Ranganathan et al., 1991; Smith et al., 1991a). Loss of eye-PKC function leads to severe defects in $[Ca^{2+}]_i$ recovery. Calcium responses are normalized to peak response. Shown are two inaC alleles.

Figure 3. All Measurable Changes in $[Ca^{2+}]_i$ Result from Extracellular Influx, Not Internal Release

(A) Rh1+4 cells recorded under conditions of zero external Ca^{2+} were stimulated with a 33 ms flash of weak UV light at time = 1.3 s (arrow). Note the complete lack of detectable rise in $[Ca^{2+}]_i$. The slowed kinetics of the light-activated current is consistent with zero $[Ca^{2+}]_{out}$ conditions (Hardie, 1991; Ranganathan et al., 1991).

(B) Averaged $[Ca^{2+}]_i$ following UV stimulation in four different cells under zero $[Ca^{2+}]_{out}$, further demonstrating the lack of detectable light-induced $[Ca^{2+}]_i$ release from intracellular stores.

(C–E) Rapid local solution exchange to zero $[Ca^{2+}]_{out}$ reversibly abolishes light-induced rise in $[Ca^{2+}]_i$. Under normal physiological solutions (C; 1.5 mM $[Ca^{2+}]_{out}$), UV-induced electrophysiological responses are accompanied by a rise in $[Ca^{2+}]_i$ (also see Figure 2). However, local perfusion of zero $[Ca^{2+}]_{out}$ solution (D) using pressure ejection from a large bore pipette eliminates the light-induced rise in $[Ca^{2+}]_i$. Reintroduction of 1.5 mM $[Ca^{2+}]_{out}$ conditions (E) restores the light-dependent change in $[Ca^{2+}]_i$. Recordings were made at V_{hold} = −40 mV.

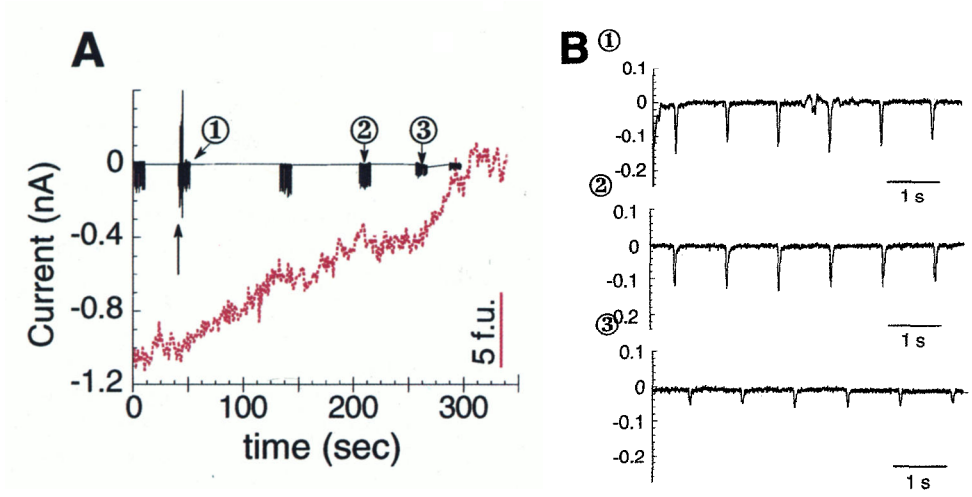


Figure 4. Application of Thapsigargin Causes Ca^{2+} Release but Does Not Mimic or Suppress the Light Response

(A) Thapsigargin (1 μM) was added to the bath at t = 40 s (indicated by the upwards arrow). This application resulted in a steady elevation of $[Ca^{2+}]_i$, indicating the presence of thapsigargin-sensitive stores. However, electrophysiological recordings of current fluxes demonstrate no change from baseline conductance resulting from depletion of these stores.

(B) Shown are electrical responses to green test flashes at t = 45, 205, and 265 s, respectively. Although the light responses are reduced in amplitude, they are not abolished, even with saturation of the thapsigargin-induced elevation in $[Ca^{2+}]_i$. Recordings were carried out at V_{hold} = −40 mV, in physiological solutions with 1.5 mM $[Ca^{2+}]_{out}$.

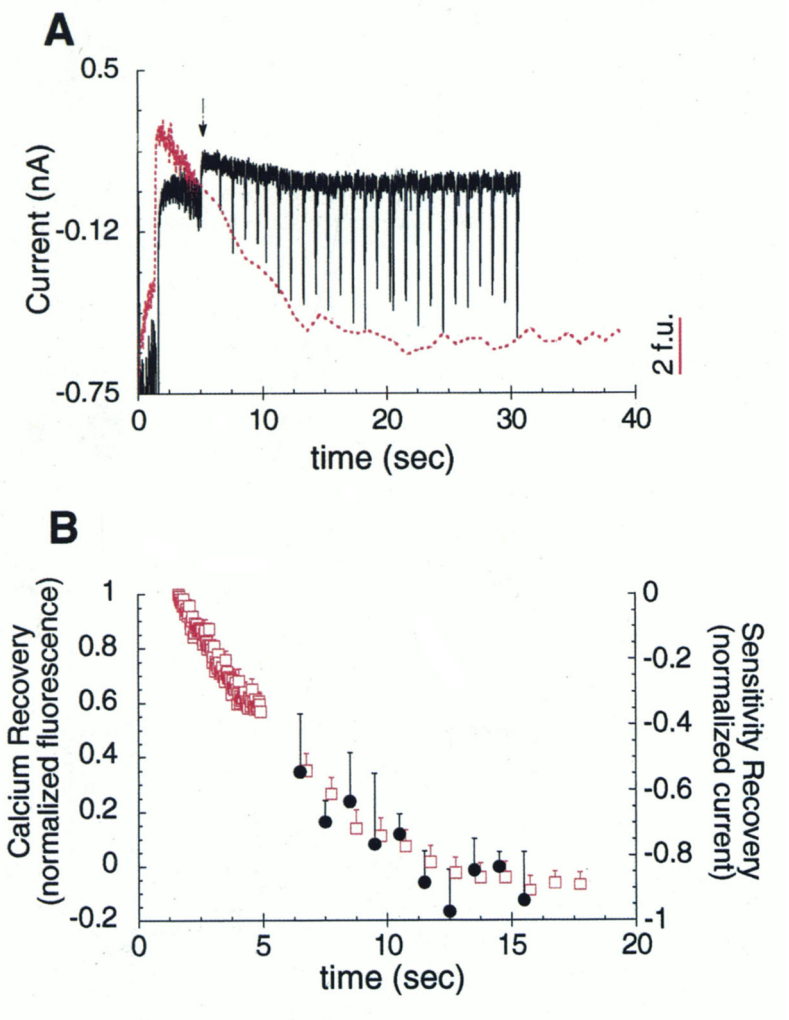


Figure 5. Kinetics of Recovery of Average $[Ca^{2+}]_i$ Matches Recovery from Adaptation

(A) Rh1+4 cells were stimulated with a 33 ms adapting UV flash as described in Experimental Procedures. Shown is the Ca^{2+} and current traces following termination of the light response (arrow). The recovery and kinetics of adaptation were monitored by giving the cells weak non-adapting green test flashes that report photoreceptor sensitivity (see Experimental Procedures). Shown are the responses to the green flashes (33 ms duration) delivered at 1 s frequency. The same flashes were also used to measure $[Ca^{2+}]_i$. The recovery of photoreceptor sensitivity correlates well with the recovery of $[Ca^{2+}]_i$ to baseline.

(B) Shown is the average recovery of $[Ca^{2+}]_i$ in four different cells, reported as a fractional increase over preflash levels and normalized to peak fluorescence (± SD). Superimposed in this graph are the average test flash response amplitudes in the same cells, expressed as a percentage of the fully recovered amplitude (closed circles). This analysis demonstrates good correlation of the rate of recovery of photoreceptor sensitivity and $[Ca^{2+}]_i$. Recording conditions were as described in Experimental Procedures. Error bars represent SD.

adaptation, suggesting that the kinetics of photoreceptor adaptation might be controlled by the dynamics of $[Ca^{2+}]_i$ (see also Levy and Fein, 1985; Walz, 1992).

To test further the possibility that changes in average $[Ca^{2+}]_i$ mediate adaptation, we asked whether experimental conditions in which the recovery of $[Ca^{2+}]_i$ is disrupted lead to defects in photoreceptor adaptation. To prevent the normal recovery of $[Ca^{2+}]_i$, we recorded from Rh1+4 photoreceptors in the presence of Cs^+ and in the absence of external Na^+, a manipulation that abolishes extrusion of $[Ca^{2+}]_i$ by inhibiting the Na^+/Ca^{2+} exchanger (Lagnado and McNaughton, 1990). In this case, repeated UV stimulation should produce step-like increases in average $[Ca^{2+}]_i$. Indeed, Figure 6 shows that replacement of all external Na^+ with Cs^+ results in light-induced elevations in $[Ca^{2+}]_i$ that do not fully return to baseline. Remarkably, though each flash of light causes a step-like increase in average $[Ca^{2+}]_i$, electrophysiological recordings demonstrate that photoreceptor adaptation nevertheless recovers fully after each stimulus (Figure 6, boxes). These results demonstrate that there is no functional link between average $[Ca^{2+}]_i$ and adaptation recovery mechanisms and highlight the fundamental distinction between average Ca^{2+} levels in the cytosol and the modulation of the signaling machinery. In contrast, inhibition of $[Ca^{2+}]_i$ re-uptake by thapsigargin causes a maintained adapted state (Figure 4). A model that explains these results is that light-dependent changes in $[Ca^{2+}]_i$ relevant for photoreceptor adaptation occur in a highly localized subcellular compartment whose recovery kinetics depends on local diffusion or re-uptake into internal stores. Indeed, we demonstrate below that spatially localized transients of Ca^{2+} are present in Drosophila photoreceptor cells, and they are responsible for mediating negative regulation of phototransduction.

Light-Dependent Ca^{2+} Influx Creates Spatially Localized Transients

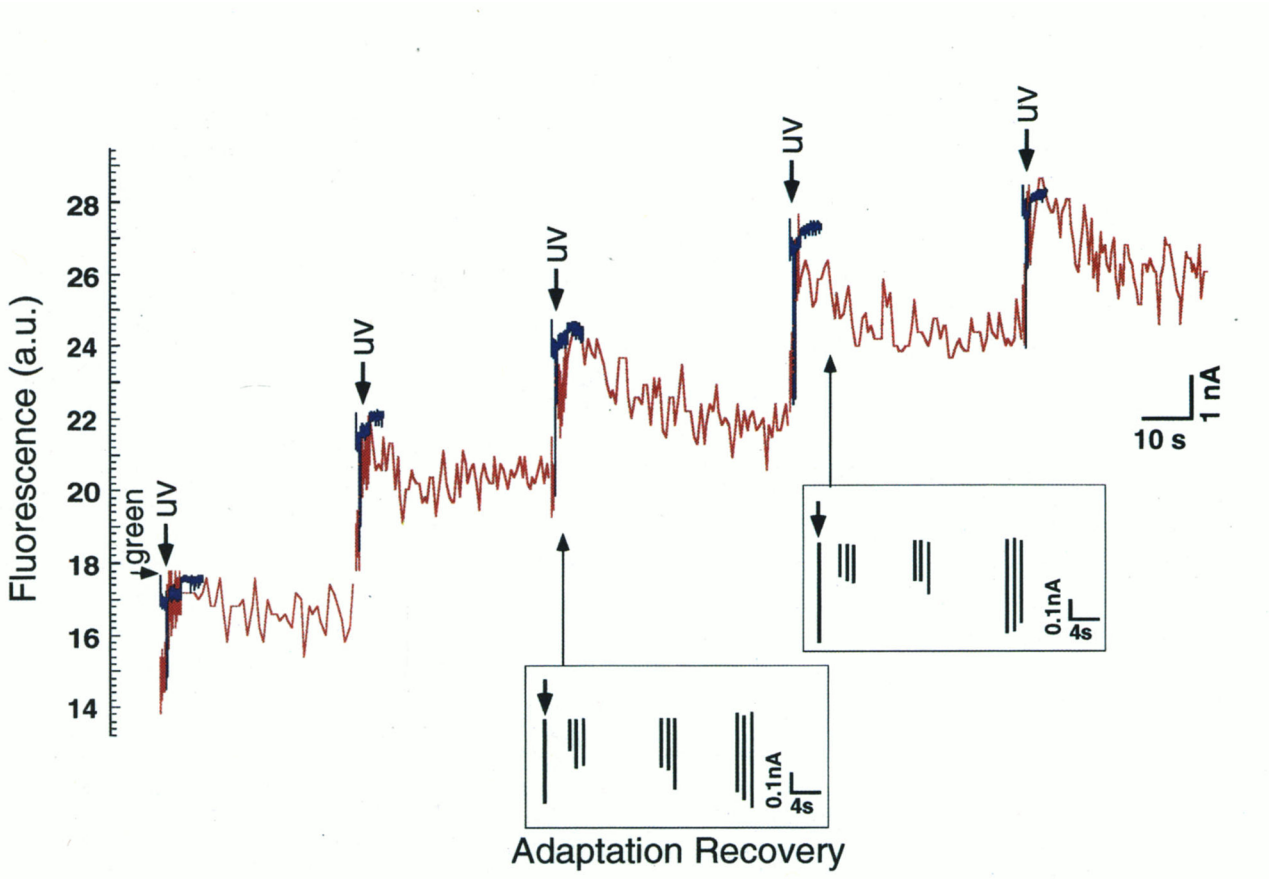
To define the spatial dynamics of light-induced Ca^{2+} changes, we imaged $[Ca^{2+}]_i$ in Rh1+4 photoreceptors at the highest practical spatial and temporal resolution possible on our confocal microscope and carried out repeated trials of weak intensity UV flashes. Several such trials were averaged temporally, and the resulting images ratioed to a preflash image to reflect relative changes in $[Ca^{2+}]_i$. Figure 7 shows a series of images from such a cell showing a clearly identifiable rhabdomere. At the time of the UV flash ($t = 0$), $[Ca^{2+}]_i$ is still at preflash levels throughout the cell. Remarkably, the first response to the UV stimulus is seen as a highly localized elevation in $[Ca^{2+}]_i$ at the junction of the rhabdomere and cell body, a finding consistent with localization of light-sensitive channels at the base of the rhabdomeres. This initial response is followed rapidly in the subsequent frames by a generalized increase in $[Ca^{2+}]_i$ at the rhabdomere, which generates a dramatic gradient of Ca^{2+} levels within the cell (note that Ca^{2+} levels also rise in the cell body, but to a much lesser degree). These findings raise the possibility that compartmentalization of $[Ca^{2+}]_i$ changes may be important in photoreceptor function.

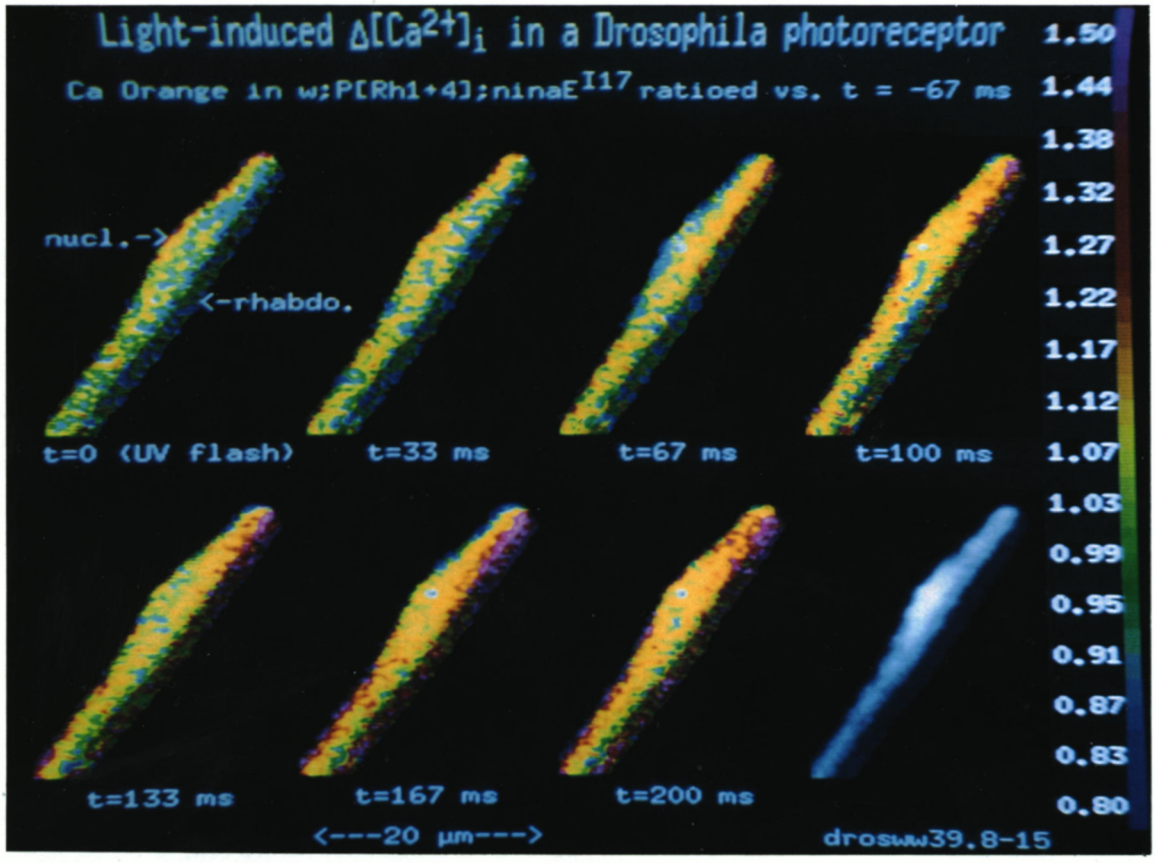
The High Transients of Ca^{2+} Are Required for Regulation of Phototransduction

What is the functional role in phototransduction of these localized transients of $[Ca^{2+}]_i$? To demonstrate that the Ca^{2+} influx generates a transient which is essential for photoreceptor function, we tested the effect of different intracellular Ca^{2+} chelators on the light and Ca^{2+} response. Figures 8C and 8D show that intracellular dialysis of Rh1+4 cells with 2 mM BAPTA, a Ca^{2+} buffer with a $K_D(Ca^{2+}) = 100$ nM (Tsien, 1980), resulted in a nearly complete loss of negative regulation. This is demonstrated by the presence of large, nondeactivating currents in response to even weak, brief flashes of UV light. These maintained currents showed no recovery in the time scale of our experiments and caused full saturation of the excitation machinery, as no further light responses could be elicited from these photoreceptors. As a medium-affinity Ca^{2+} chelator, we chose Trans-5, $K_D(Ca^{2+}) = 6.0\ \mu M$ (Adams et al., 1988), a novel compound with close homology to BAPTA (only a subtle stereochemical alteration is responsible for a 60-fold decrease in Ca^{2+} affinity). Unlike the APTRA series of chelators, in which the BAPTA binding site is severely truncated, Trans-5 retains high selectivity for Ca^{2+} over Mg^{2+}. Trans-5 also avoids the potential photochemical complications caused by nitro substituents in chelators such as 5-nitro-BAPTA. Figure 8E demonstrates that application of Trans-5 also results in the presence of large, nondeactivating currents that show no recovery in the time scale of our experiments. In contrast, recordings in the presence of the Ca^{2+} buffer Anis-1 ($Kd(Ca^{2+}) = 1.8$ mM; Irving and da Silva, 1963) produced responses indistinguishable from those of wild-type photoreceptors (compare Figures 8A and 8F). Together, these results demonstrate that the light-dependent Ca^{2+} influx generates micromolar range, highly localized transients that are essential to drive negative regulation.

An Eye-Specific Protein Kinase C Is Required for Ca^{2+} Recovery following Activation of the Visual Cascade

Since localized $[Ca^{2+}]_i$ drives critical aspects of photoreceptor cell regulation, the molecular mechanisms that determine the spatial and temporal dynamics of cytosolic Ca^{2+} are of great interest. We asked whether any Drosophila phototransduction mutants known to be defective in regulation also have altered Ca^{2+} recovery kinetics. In wild-type Rh1+4 photoreceptors, the kinetics of $[Ca^{2+}]_i$ recovery following a UV flash is far slower than the rate of deactivation of the light-activated current (Figures 2A and 2B). Exponential fits to these data showed current deactivation time constants of 33.7 ± 10.9 ms and Ca^{2+} recovery time constants of 30.2 ± 4.2 s. The kinetics of Ca^{2+} recovery is remarkably conserved between cells recorded under


Fluorescence (a.u.)
28
26
24
22
20
18
16
14
green
UV
UV
UV
UV
UV
1 nA
10 s
0.1nA
4s
0.1nA
4s
Adaptation Recovery


Light-induced Δ[Ca2+]i in a Drosophila photoreceptor
Ca Orange in w;P[Rh1+4];ninaE I17 ratioed vs. t = -67 ms
nucl.->
<-rhabdo.
t=0 (UV flash)
t=33 ms
t=67 ms
t=100 ms
t=133 ms
t=167 ms
t=200 ms
<---20 μm--->
drosww39.8-15
1.50
1.44
1.38
1.32
1.27
1.22
1.17
1.12
1.07
1.03
0.99
0.95
0.91
0.87
0.83
0.80

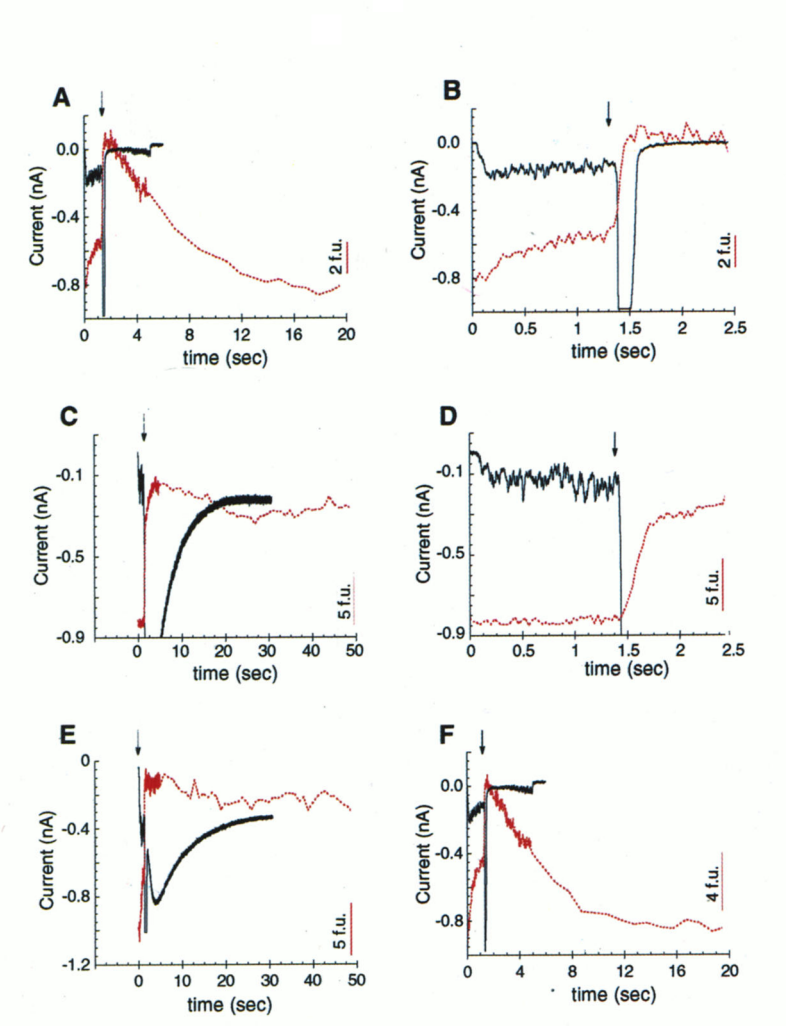


Figure 8. Localized Transients of $[Ca^{2+}]_i$ of Micromolar Amplitude Are Required for Negative Regulation

(A and B) Electrical and $[Ca^{2+}]_i$ responses in a Rh1+4 photoreceptor cell with 1.5 mM $[Ca^{2+}]_{out}$. (B) shows an expanded time scale. Recording conditions are as described in Experimental Procedures.

(C and D) Addition of 2 mM BAPTA ($K_D(Ca^{2+})$ = 100 nM) to internal solution destroys negative regulation, resulting in large, noninactivating electrical responses and maintained elevations in $[Ca^{2+}]_i$. The light response deactivates within 200 ms in control cells (B), whereas it takes over 30 s in the presence of BAPTA. Note the slowed rise of $[Ca^{2+}]_i$, consistent with the buffering action of BAPTA (D).

(E) Addition of 2 mM Trans-5 ($K_D(Ca^{2+})$ = 6.0 μM) to internal solutions shows a similar phenotype as with BAPTA, demonstrating that $[Ca^{2+}]_i$ transients must rise to several micromolar.

(F) Addition of 4 mM Anis-1 ($K_D(Ca^{2+})$ = 1.5 mM) to internal solutions has no effect, indicating that $[Ca^{2+}]_i$ transients do not rise to the millimolar level. All recordings were at V_{hold} = −40 mV and in physiological solutions, except for the presence of internal Ca^{2+} buffers as noted. Arrows indicate the location of the UV flash.

similar conditions (Figure 2C), consistent with a finely regulated Ca^{2+} extrusion/uptake process. Figure 2D shows that mutants defective in eye-PKC (Ranganathan et al., 1991; Smith et al., 1991a) display a dramatic slowed recovery of light-induced $[Ca^{2+}]_i$ (time constants of 111.1 ± 9.5 s versus 30.2 ± 4.2 s). Previously, we showed that eye-PKC function is required for termination of the light response and that entry of extracellular Ca^{2+} is required for activation of this PKC (Smith et al., 1991a). Thus, light-activated opening of membrane channels leads to entry of Ca^{2+}, which then activates eye-PKC to mediate response deactivation.

Figure 6. Dissociation of Average $[Ca^{2+}]_i$ and Adaptation: A Requirement for $[Ca^{2+}]_i$ Compartmentalization

Photoreceptors were recorded in the presence of extracellular Cs^+ and in the absence of Na^+ to block the Na^+/Ca^{2+} exchanger (see Experimental Procedures). Shown are 200 s of $[Ca^{2+}]_i$ recordings, with UV flashes at 10, 40, 80, 120, and 160 s, respectively. The recovery and kinetics of adaptation were analyzed as described in Experimental Procedures. In the presence of Cs^+, each UV flash leads to a step-like increase in average $[Ca^{2+}]_i$ (see text for details). However, photoreceptors still respond normally to each UV flash and recover from the adapting UV stimulus at each new level of $[Ca^{2+}]_i$. The boxed areas display enlargements of the adaptation test: shown are responses to the green test flashes before (arrow) and after the UV stimulation, demonstrating full recovery of sensitivity.

Figure 7. Spatial Localization of Light-Induced Ca^{2+} Transients in Rh1+4 Photoreceptors

Shown are sequential images of a cell in response to a 33 ms UV stimulus at t = 0. Ca^{2+} changes relative to preflash levels are displayed using a pseudocolor scale. Images were acquired at video frame rate (30 Hz). The rhabdomere of this cell (rhabdo) is distinctly resolved from the cell body (nucl. = nucleus). By the third image (t = 67 ms), the first response in $[Ca^{2+}]_i$ is noted as a highly localized increase at the base of the rhabdomere. This time window (33–67 ms) is consistent with the time latency of the light response (Ranganathan et al., 1994). The subrhabdomeric Ca^{2+} burst is followed rapidly by a wave of elevated $[Ca^{2+}]_i$ largely localized to the rhabdomere. The $[Ca^{2+}]_i$ in the cell body also rises, but to a lesser degree. To enhance signals above noise, we time averaged images from five identical flash trials, and each resulting image was subjected to 3 pixel × 3 pixel spatial averaging. This pixel averaging does not reduce our spatial resolution, since pixel sizes are much smaller than optical resolution at this magnification (see scale bar). The black and white photograph in the bottom, right hand panel shows the same cell at high contrast levels to emphasize the difference between cell body and rhabdomere.

Recent studies of the role of eye-PKC in photoreceptor cell adaptation were interpreted to suggest that this protein functions to limit the rise of $[Ca^{2+}]_i$ through inhibition of intracellular Ca^{2+} release mechanisms (Hardie et al., 1993). Our data support an alternative model in which eye-PKC acts to enhance the rate of removal of intracellular Ca^{2+}. Thus, eye-PKC functions in a regulatory loop in which Ca^{2+} entry is required for its activation, and once activated, it regulates the kinetics of Ca^{2+} recovery (negative feedback). A genetic screen for enhancers and suppressors of eye-PKC mutants may identify the molecular components that generate and modulate the spatial and temporal patterns of $[Ca^{2+}]_i$ in Drosophila photoreceptors.

Concluding Remarks

The present studies have revealed a hierarchy of $[Ca^{2+}]_i$ localization in Drosophila photoreceptors. High speed confocal imaging with a spatial resolution of about 1 μm and a temporal resolution of 33 ms demonstrated that influx of Ca^{2+} through light-activated channels causes $[Ca^{2+}]_i$ to rise earlier and higher in the rhabdomeres than in the rest of the cell. Rhabdomeres consist of tightly packed microvilli, approximately 1–2 μm in length and 30–50 nm wide, so that all parts of the rhabdomere are within 25 nm of the plasma membrane. Thus, the study of light-induced $[Ca^{2+}]_i$ dynamics in this organelle offers the unique opportunity of studying large-amplitude submembrane Ca^{2+} changes during signal transduction. The subcellular distribution of light-activated channels was not previously known. The finding that they are concentrated in the rhabdomeres makes functional sense because the transduction molecules are then clustered for maximum speed of response. This is particularly important to photoreceptor neurons, which rely on very fast temporal resolution to resolve closely spaced visual stimuli.

The existence of functionally relevant, ultramicro domains of high $[Ca^{2+}]_i$ has been postulated in a number of signaling process (reviewed by Augustine and Neher, 1992a). Theoretical calculations indicate that $[Ca^{2+}]_i$ can reach tens to hundreds of micromolar within 10–100 nm from each conducting channel. Such ultramicro domains would not only be too small to be resolved by present optical techniques but reach $[Ca^{2+}]_i$ levels far above the sensitivity range of typical fluorescent indicators (Tsien, 1989). In this work, we have demonstrated the existence and physiological importance of ultramicro domains in Drosophila photoreceptors, as shown by the profound effect of the medium affinity chelator Trans-5, which blocked deactivation of the light response (negative regulation) as effectively as the much higher affinity chelator BAPTA. If negative regulation were controlled directly by the submicromolar to low micromolar $[Ca^{2+}]_i$ levels that Ca-orange can resolve, then the staircase of measured $[Ca^{2+}]_i$ in Figure 6 should have caused cumulative increases in adaptation, and Trans-5 should have had too low an affinity to buffer $[Ca^{2+}]_i$ and affect response kinetics significantly. Instead, negative regulation seems to result from submicroscopically localized $[Ca^{2+}]_i$ transients that are comparable to or higher than 6 μM, the Trans-5 K_D for Ca^{2+}. Together, these results provide experimental evidence for a functional requirement of spatially localized $[Ca^{2+}]_i$ in intracellular signaling.

The Drosophila phototransduction cascade is considered one of the best genetic models of phosphoinositide-mediated signal transduction. In this study, we demonstrate the presence of internal Ca^{2+} stores pharmacologically consistent with IP_3 sensitivity but show that these stores are not required for the light response. Thus, Drosophila photoexcitation may depend on IP_3-sensitive Ca^{2+} stores with novel pharmacological properties rendering them insensitive to thapsigargin or may depend on a non-IP_3-sensitive store. Alternatively, photoexcitation may be mediated through direct action of inositol phosphates or phosphatidylinositol bis-phosphate breakdown. It should now be possible to test these models rigorously using a combination of $[Ca^{2+}]_i$ measurement, electrophysiological recording, and pharmacological and genetic manipulation of the photoreceptor cell environment.

Experimental Procedures

DNA Constructs and Transgenic Animals

Rh1+4 consists of the entire structural gene for the UV-sensitive Rh4 rhodopsin under the control of the Rh1 promoter. The constructs were generated as described (Feiler et al., 1992). The modified opsins were subcloned into a P element transformation vector containing the *rosy*$^+$ gene as a selectable marker (Karess and Rubin, 1984) and introduced into animals carrying a deletion of the endogenous Rh1 rhodopsin gene. Drosophila P element-mediated germ-line transformation was carried out as described (Karess and Rubin, 1984). Multiple independent lines were obtained and subjected to expression studies by biochemical, physiological, and Western blot analyses. The R state of Rh4 has an absorption maxima at 375 nm, and the M state has maxima at 465 nm (Feiler et al., 1992).

Fly Stocks

inaC alleles were originally obtained from W. Pak and characterized in this laboratory (Smith et al., 1991a). *ninaE*^{117} stock is a null allele of Rh1 (O'Tousa et al., 1985; Zuker et al., 1985). The wild-type stock used in these studies is *w*^{1118}. All stocks were constructed using standard balancer stocks (Lindsley and Zimm, 1992).

Electrophysiological Recordings

Preparations of isolated Drosophila photoreceptors and patch-clamp methodology were as previously described (Ranganathan et al., 1991). Recordings were made in bath solution containing 120 mM NaCl, 4 mM KCl, 1.5 mM $CaCl_2$, 10 mM HEPES–KOH, 5 mM proline, and 25 mM sucrose (pH 7.15). Internal solution contained 90 mM potassium gluconate, 45 mM KCl, 10 mM HEPES–KOH, 2 mM $MgCl_2$, 3 mM Mg-ATP, 0.5 mM Na-GTP, and 0.2 mM Ca-orange (Molecular Probes). For experiments blocking the Na^+/Ca^{2+} exchanger, recordings were made in bath solutions in which Na^+ was replaced by 120 mM CsCl. For the experiments described in Figures 3C–3E, the local perfusion consisted of 124 mM NaCl, 4 mM KCl, 8 mM $MgCl_2$, 10 mM HEPES–KOH, 5 mM proline, and 25 mM sucrose (pH 7.15).

Photoreceptors were excited with UV light at 351/364 nm as described in Confocal Microscopy. All cells were dark-adapted prior to whole-cell recordings and were matched for sensitivity by monitoring quantum bump generation. Typical stimulating

light produced nonsaturating electrical responses under physiological solutions. For quantitation of photoreceptor deactivation, time constants for current relaxation were measured from single exponential fits to tail currents of the light response (Dolph et al., 1993). A minimum of four different cells were characterized in each case.

Confocal Microscopy

Fluorescence imaging was performed on a custom-made confocal microscope enabling full frame image acquisition at video rates (Tsien and Bacskai, 1994). A HeNe laser operating at a wavelength of 543.5 nm (GreNe, Melles Griot) was used as the excitation source to illuminate Ca-orange (Molecular Probes), and fluorescence emission was detected at >560 nm by placing a barrier filter (Omega Optical) in front of the photomultiplier tube. The confocal pinhole was large and generally allowed optical sectioning as thick as the cell to maximize collection efficiency and minimize the excitation power needed. An argon laser (Coherent) operating in the ultraviolet (principally 351 and 364 nm) was used as the stimulus source and was gated with an electromechanical shutter (Newport) controlled by the imaging computer. A Nikon 40× (NA = 1.3) or 60× (NA = 1.4) oil immersion objective was used on an inverted microscope (Zeiss, IM35). Single wavelength images were acquired and stored on an optical memory disk (Panasonic TQ2028F) at up to 30 frames per second. Time course plots were generated by averaging the pixel intensities within the entire cell for successive video images. Pseudocolor images were generated from the single wavelength images by calculating a ratio of fluorescence versus an arbitrarily defined time zero. The resulting ratios were pseudocolored from blues to reds to indicate relative changes in $[Ca^{2+}]_i$ from low to high, respectively, and then digitally smoothed with a 3 × 3 convolution kernel.

Acknowledgments

We thank Stephen Adams for the synthesis of Trans-5. We also thank Robert Hardy for help generating various genetic combinations of Rh1+4 flies and Kristin Scott and Maurice Kernan for helpful discussions. R. R. was supported by a training grant from the Medical Scientist Training Program. C. S. Z. acknowledges support from the Pew Foundation. C. S. Z. and R. Y. T. are investigators of the Howard Hughes Medical Institute.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *"advertisement"* in accordance with 18 USC Section 1734 solely to indicate this fact.

Received July 6, 1994.

References

Adams, S. R., Kao, J. P. Y., Grynkiewicz, G., Minta, A., and Tsien, R. Y. (1988). Biologically useful chelators that release Ca^{2+} upon illumination. J. Am. Chem. Soc. *110*, 3212–3220.

Augustine, G. J., and Neher, E. (1992a). Neuronal Ca^{2+} signalling takes the local route. Curr. Opin. Neurobiol. *2*, 302–307.

Augustine, G. J., and Neher, E. (1992b). Calcium requirements for secretion of bovine chromaffin cells. J. Physiol. *450*, 247–271.

Berridge, M. (1993). Inositol trisphosphate and calcium signaling. Nature *361*, 315–325.

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

Brown, J. (1986). Calcium and light adaptation in invertebrate photoreceptors. In The Molecular Mechanisms of Photoreception, H. Stieve, ed. (Berlin: Springer-Verlag), pp. 231–240.

Brown, J., and Blinks, J. (1974). Changes in intracellular free calcium concentration during illumination of invertebrate photoreceptors. Detection with aequorin. J. Gen. Physiol. *64*, 643–665.

Brown, J., Brown, P., and Pinto, L. (1977). Detection of light-induced changes of intracellular ionized calcium concentration in Limulus ventral photoreceptors using arsenazo III. J. Physiol. *267*, 299–320.

Britt, S. G., Feiler, R., Kirschfeld, K., and Zuker, C. S. (1993). Spectral tuning of rhodopsin and metarhodopsin in vivo. Neuron *11*, 29–39.

Brundage, R. A., Fogarty, K. E., Tuft, R. A., and Fay, F. S. (1993). Chemotaxis of newt eosinophils: calcium regulation of chemotactic response. Am. J. Physiol. *265*, C1527–C1543.

Chad, J., and Eckert, R. (1984). Calcium domains associated with individual channels can account for anomalous voltage relations of Ca-dependent responses. Biophys. J. *45*, 993–999.

Cheng, H., Lederer, W., and Cannell, M. (1993). Calcium sparks: elementary events underlying excitation–contraction coupling in heart muscle. Science *262*, 740–742.

Dolph, P. J., Ranganathan, R., Colley, N. J., Hardy, R. W., Socolich, M., and Zuker, C. S. (1993). Arrestin function in inactivation of G protein–coupled receptor rhodopsin in vivo. Science *260*, 1910–1916.

Feiler, R., Harris, W. A., Kirschfeld, K., Wehrhahn, C., and Zuker, C. S. (1988). Targeted misexpression of a *Drosophila* opsin gene leads to altered visual function. Nature *333*, 737–741.

Feiler, R., Bjornson, R., Kirshfeld, K., Mismer, D., Rubin, G. M., Smith, D. P., Socolich, M., and Zuker, C. S. (1992). Ectopic expression of ultraviolet-rhodopsins in the blue photoreceptor cells of Drosophila: visual physiology and photochemistry of transgenic animals. J. Neurosci. *12*, 3862–3868.

Fogelson, A., and Zucker, R. (1985). Presynaptic calcium diffusion from various arrays of single channels. Implications for transmitter release and synaptic facilitation. Biophys. J. *48*, 1003–1007.

Franceschini, N. (1979). Voltage clamp by light. Invest. Ophtalmol. (Suppl.), 5.

Guthrie, P., Segal, M., and Kater, S. (1991). Independent regulation of calcium revealed by imaging dendritic spines. Nature *354*, 76–80.

Hardie, R. (1983). Progress in Sensory Physiology. (New York: Springer-Verlag).

Hardie, R. (1991). Whole-cell recordings of the light induced current in dissociated *Drosophila* photoreceptors: evidence for feedback by calcium permeating the light-sensitive channels. Proc. R. Soc. Lond. (B) *245*, 203–210.

Hardie, R., and Minke, B. (1992). The *trp* gene is essential for a light-activated Ca^{2+} channel in Drosophila photoreceptors. Neuron *8*, 643–651.

Hardie, R. C., and Minke, B. (1993). Novel Ca^{2+} channels underlying transduction in *Drosophila* photoreceptors: implications for phosphoinositide-mediated Ca^{2+} mobilization. Trends Neurosci. *16*, 371–376.

Hardie, R. C., Peretz, A., Suss-Toby, E., Rom-Glas, A., Bishop, S. A., and Selinger, Z. (1993). Protein kinase C is required for light adaptation in *Drosophila* photoreceptors. Nature *363*, 634–637.

Hernandez-Cruz, A., Sala, F., and Adams, P. (1990). Subcellular calcium transients visualized by confocal microsocope in a voltage clamped vertebrate neuron. Science *247*, 858–862.

Hotta, Y., and Benzer, S. (1970). Genetic dissection of the *Drosophila* nervous system by means of mosaics. Proc. Natl. Acad. Sci. USA *67*, 1156–1163.

Irving, H., and da Silva, J. J. R. F. (1963). The stabilities of metal complexes of some derivatives of iminodiacetic acid. J. Chem. Soc., 3308–3320.

Karess, R. E., and Rubin, G. M. (1984). Analysis of P transposable element functions in Drosophila. Cell *38*, 135–146.

Lagnado, L., and McNaughton, P. A. (1990). Electrogenic properties of the Na:Ca exchange. J. Membr. Biol. *113*, 177–191.

Lee, Y.-J., Dobbs, M. B., Verardi, M. L., and Hyde, D. R. (1990). *dgq*: a Drosophila gene encoding a visual system-specific G_α

molecule. Neuron *5*, 889–898.

Levy, S., and Fein, A. (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.

Levy, S., and Payne, R. (1993). A lingering elevation of Cai accompanies inhibition of inositol 1,4,5-trisphosphate-induced Ca release in Limulus ventral photoreceptors. J. Gen. Physiol. *101*, 67–84.

Lindsley, D. L., and Zimm, G. G. (1992). Genome of *Drosophila melanogaster*. (San Diego, California: Academic Press).

Meyer, T. (1991). Cell signaling by second messenger waves. Cell *64*, 675–678.

Minke, B., and Selinger, Z. (1991). Inositol lipid pathway in fly photoreceptors: excitation, calcium mobilization and retinal degeneration. Prog. Ret. Res. *11*.

Muller, W., and Connor, J. (1991). Dendritic spines as individual neuronal compartments for synaptic Ca^{2+} responses. Nature *354*, 73–76.

Nagy, K., and Stieve, H. (1983). Changes in intracellular free calcium ion concentration in the course of light adaptation measured by arsenazo III in the Limulus photoreceptors. Biophys. Struct. Mech. *9*, 207–223.

Nowycky, M. C., and Pinter, M. J. (1993). Time courses of calcium and calcium-bound buffers following calcium influx in a model cell. Biophys. J. *64*, 77–91.

O'Day, P., and Gray-Keller, M. (1989). Evidence for electrogenic Na/Ca exchange in Limulus ventral photoreceptors. J. Gen. Physiol. *93*, 473–492.

O'Tousa, J. E., Baehr, W., Martin, R. L., Hirsh, J., Pak, W. L., and Applebury, M. L. (1985). The Drosophila *ninaE* gene encodes an opsin. Cell *40*, 839–850.

Pak, W. L., Grossfield, J., and Arnold, K. (1970). Mutants of the visual pathway of *Drosophila melanogaster*. Nature *227*, 518–520.

Payne, R., and Fein, A. (1987). Inositol 1,4,5 trisphosphate releases calcium from specialized sites within Limulus photoreceptors. J. Cell Biol. *104*, 933–937.

Peretz, A., Suss-Toby, E., Rom-Glas, A., Arnon, A., Payne, R., and Minke, B. (1994). The light response of Drosophila photoreceptors is accompanied by an increase in cellular calcium: effects of specific mutations. Neuron *12*, 1257–1267.

Putney, J. W., Jr., and Bird, G. St. J. (1993). The signal for capacitative calcium entry. Cell *75*, 199–201.

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

Ranganathan, R., Malicki, D. M., and Zuker, C. S. (1994). Signal transduction in Drosphila photoreceptors. Annu. Rev. Neurosci., in press.

Ratto, G. M., Payne, R., Owen, W. G., and Tsien, R. Y. (1988). The concentration of cytosolic free calcium in vertebrate rod outer segments measured with Fura-2. J. Neurosci. *8*, 3240–3246.

Rehder, V., and Kater, S. (1992). Regulation of neuronal growth cone filopodia by intracellular calcium. J. Neurosci. *12*, 3175–3186.

Sandler, C., and Kirschfeld, K. (1991). Light-induced extracellular calcium and sodium concentration changes in the retina of Calliphora: involvement in the mechanism of light adaptation. J. Comp. Physiol. (A) *169*, 299–311.

Schaeffer, E., Smith, D., Mardon, G., Quinn, W., and Zuker, C. (1989). Isolation and characterization of two new Drosophila protein kinase C genes, including one specifically expressed in photoreceptor cells. Cell *57*, 403–412.

Simon, S., and Llinas, R. (1985). Compartmentalization of the submembrane calcium activity during calcium influx and its significance in transmitter release. Biophys. J. *48*, 485–498.

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

Smith, D. P., Stamnes, M. A., and Zuker, C. S. (1991b). Signal transduction in the visual system of *Drosophila*. Annu. Rev. Cell Biol. *7*, 161–190.

Thastrup, O., Cullen, P., Drobak, B., Hanley, M., and Dawson, A. (1990). Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. Proc. Natl. Acad. Sci. USA *87*, 2466–2470.

Thomas, P., Suprenant, A., and Almers, W. (1990). Cytosolic Ca^{2+}, exocytosis, and endocytosis in single melanotrophs of the rat pituitary. Neuron *5*, 723–733.

Tsien, R. Y., and Bacskai, B. J. (1994). Vide-rate confocal microscopy. In Handbook of Biological Confocal Microscopy, 2nd edition, J. Pawley, ed. (New York: Plenum Press), in press.

Tsien, R. W., and Tsien, R. Y. (1990). Calcium channels, stores, and oscillations. Annu. Rev. Cell Biol. *6*, 715–760.

Tsien, R. Y. (1980). New calcium indicators in buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. Biochemistry *19*, 2396–2404.

Tsien, R. Y. (1989). Fluorescent probes of cell signaling. Annu. Rev. Neurol. *12*, 227–253.

Walz, B. (1992). Enhancement of sensitivity in photoreceptors of the honey bee drone by light and by calcium. J. Comp. Physiol. (A) *1*, 605–613.

Walz, B., Zimmermann, B., and Seidl, S. (1994). Intracellular calcium concentration and latency of light-induced calcium changes in photoreceptors of the honeybee drone. J. Comp. Physiol. (A) *174*, 421–431.

Zheng, J., Felder, M., Connor, J., and Poo, M. (1994). Turning of nerve growth cones induced by neurotransmitters. Nature *368*, 140–144.

Zuker, C. S. (1992). Phototransduction in *Drosophila*: a paradigm for the genetic dissection of sensory transduction cascades. Curr. Opin. Neurobiol. *2*, 622–627.

Zuker, C. S., Cowman, A. F., and Rubin, G. M. (1985). Isolation and structure of a rhodopsin gene from D. melanogaster. Cell *40*, 851–858.

Note Added in Proof

R. R. is now at the following address: Department of Neurobiology, Harvard Medical School, Boston, Massachusetts 02115.