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Calmodulin Regulation of Drosophila Light-Activated Channels and Receptor Function Mediates Termination of the Light Response In Vivo

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Summary

Calmodulin (CAM) participates in a variety of intracellular transduction processes by modulating signaling molecules in response to calcium changes. We report the characterization of Drosophila *Cam* mutants and the role of CAM in photoreceptor cell function. Contrary to current models of excitation and TRP channel function, we demonstrate that the transient phenotype of *trp* mutants can be explained by CAM regulation of the TRPL channel rather than by the loss of a store-operated conductance leading to depletion of the internal stores. We also analyzed light responses in a variety of mutant and transgenic backgrounds and demonstrate the importance of calmodulin in mediating calcium-dependent negative regulation of phototransduction. Our results show that CAM coordinates termination of the light response by modulating receptor and ion channel activity.

Introduction

Calcium influx is fundamental for the activation and regulation of many cell signaling events, including those involved in cell growth, differentiation, and cellular physiology (reviewed in Tsien and Tsien, 1990; Augustine and Neher, 1992). The concentration of free calcium in the cytoplasm is normally low and becomes rapidly elevated by the opening of calcium-permeable channels in the plasma membrane or in internal stores. Calcium entry through ion channels yields a complex spatial and temporal gradient of intracellular calcium, which functions as an important regulatory signal. The molecular mechanisms of calcium action are quite diverse, as calcium ions can bind and modify the function of a wide range of cellular proteins.

Calmodulin (CAM) is one of the primary sensors of calcium changes (for review, see Klee and Vanaman, 1982; Cohen and Klee, 1988). Calmodulin is a ubiquitous calcium binding protein that regulates the activity of many target proteins when complexed with calcium. Signaling components that are modulated by CAM include kinases, phosphatases, molecular motors, nitric oxide synthase, adenylate cyclase, cGMP phosphodiesterase, and ion channels. Although biochemical approaches have identified numerous molecular targets of CAM, it has often been difficult to integrate studies on single CAM targets into a comprehensive picture of the role of calcium and CAM in a well-defined signaling cascade. Many studies relying on pharmacological agents have tightly implicated CAM in the regulation of a variety of different signaling pathways and physiological processes. These studies, however, suffer from the difficulty of assessing in vivo roles from in vitro studies. Genetic approaches offer the possibility of dissecting CAM function in vivo. In Drosophila, there is a single gene encoding CAM (Yamanaka et al., 1987; Doyle et al., 1990) and null *Cam* mutants are lethal (Heiman et al., 1996). We have taken advantage of a wealth of genetic and physiological resources to dissect the role of CAM in a prototypical phospholipase C signaling cascade.

Phototransduction in Drosophila is a model system to study phosphoinositide-mediated and calcium-regulated signal transduction events in vivo (reviewed in Ranganathan et al., 1995). This pathway is amenable to molecular and genetic dissection, and many of the components of this cascade have been isolated and characterized. In addition, phototransduction offers the advantage of resolving signaling events with exquisite sensitivity (activation of single receptor molecules) and speed (response times of less than 100 milliseconds). In Drosophila photoreceptor neurons, light activation of rhodopsin activates a heterotrimeric G protein (Lee et al., 1990; Scott et al., 1995), which in turn activates phospholipase C (PLC) (Yoshioka et al., 1983; Bloomquist et al., 1988). PLC catalyzes the hydrolysis of the minor membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP_2) into the second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Berridge, 1987). Activation of PLC then leads to the opening of cation-selective membrane channels encoded by the *transient receptor potential* (*trp*) (Montell and Rubin, 1989; Hardie and Minke, 1992) and *trp-like* (*trpl*) genes (Phillips et al., 1992; Niemeyer et al., 1996) and a transient increase in intracellular calcium. Although the exact mechanism of gating of the light-activated channels remains unsolved, current models revolve around calcium and internal stores (Hardie and Minke, 1995; Minke and Selinger, 1996; Xu et al., 1997; but see Acharya et al., 1997).

Calcium mediates positive and negative regulation of the visual cascade (Hardie, 1991; Ranganathan et al., 1991). Light-induced activation of TRP and TRPL ion channels leads to the entry of extracellular calcium and the calcium-dependent regulation of the light response. Indeed, most (Arnon et al., 1997) or all (Peretz et al., 1994; Ranganathan et al., 1994; Hardie, 1996) detectable changes in calcium during phototransduction result from calcium entry through TRP and TRPL, suggesting that activity-dependent feedback is the primary function of calcium. Calcium has also been proposed to be necessary for excitation because intracellular calcium is required for photoreceptor cell responsiveness (Hardie et al., 1993), and *trp* mutants lacking the major calcium-permeable TRP ion channel cannot maintain light responses (Hardie and Minke, 1992). These findings have

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led to the hypothesis that calcium release from internal stores is required for activation of the phototransduction cascade and that the TRP channel functions as a store-operated channel gated by the light-induced emptying of the internal stores (Hardie and Minke, 1995; Minke and Selinger, 1996).

Given the critical role of calcium in this pathway, many studies have sought to determine if CAM functions in this signaling cascade, and several potential targets of CAM have been identified. The TRP and TRPL light-activated ion channels bind CAM in vitro: TRP contains a single calmodulin binding site in its carboxyl terminus (Phillips et al., 1992; Chevesich et al., 1997) and TRPL contains two sites (Phillips et al., 1992; Warr and Kelly, 1996). The eye-specific NINAC kinase binds CAM in vitro (Porter et al., 1995), and *ninaC* mutants show CAM mislocalization and light responses with deactivation defects (Porter et al., 1993; Hofstee et al., 1996). The visual arrestins may also be regulated by CAM. Arrestins are crucial components in the deactivation cycle of G protein–coupled receptors, and the Drosophila visual arrestins are phosphorylated by a calcium/calmodulin-dependent kinase in response to light (LeVine et al., 1990; Matsumoto and Yamada, 1991; Matsumoto et al., 1994; Kahn and Matsumoto, 1997). Finally, it has recently been reported that CAM regulates the ryanodine receptor in photoreceptor neurons (Arnon et al., 1997). Despite the multiple targets for CAM, the functional relevance of these associations has not been established.

We now report the characterization of Drosophila *Cam* mutants and the role of CAM in the regulation of photoreceptor cell function. We use a combination of genetics, transgenic animals, and electrophysiology to show that CAM functions as a primary transducer of calcium changes in vivo. We characterized macroscopic and quantal responses and demonstrate that CAM regulation of receptor function and ion channels orchestrates the termination of the light response.

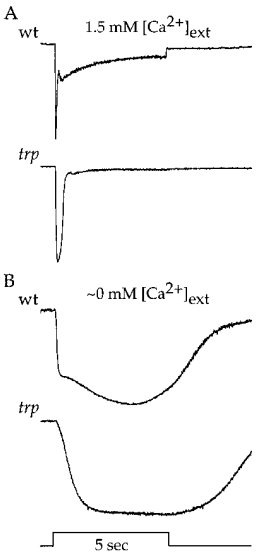

Figure 1. Light Responses in *trp* Are Not Transient in the Absence of Calcium

Shown are representative whole-cell, voltage-clamp recordings of light-activated currents from wild-type and *trp* mutant cells in the presence of 1.5 mM extracellular calcium (A) or in the absence of added calcium (B). Equivalent results were obtained in the presence 8 mM $MgCl_2$ in the bath solution. Recordings were carried out as described in Experimental Procedures, and cells were stimulated with 5 s pulses of 580 nm light (log [I] = −1). Current traces are normalized to the peak amplitude. Note that while *trp* responses decay to baseline in the presence of calcium, they do not decay in the absence of calcium. Similar results were obtained for at least seven cells of each genotype in each calcium concentration. Cells were placed in zero extracellular calcium and analyzed within 5–20 min to prevent cellular rundown. The logic of this strategy is supported by the observation that lanthanum blockage of the TRP channel instantaneously prevents calcium entry and phenocopies the *trp* phenotype (Hardie and Minke, 1992; Niemeyer et al., 1996).

Results and Discussion

CAM Regulates the TRPL Ion Channel

Drosophila mutants lacking the TRP ion channel display a transient response to light during prolonged and strong stimulation (thus, the name *transient receptor potential*) (Cosens and Manning, 1969) (Figure 1A). Because *trp* mutant photoreceptors cannot sustain a steady-state current, it has been proposed that an excitatory factor becomes depleted in these cells (Hardie and Minke, 1992, 1995). Since the TRP ion channel is the major calcium-permeable channel in photoreceptors (Hardie and Minke, 1992) and because intracellular calcium is required for photoreceptor cell responsiveness (Hardie et al., 1993), it has been proposed that loss of calcium entry through TRP leads to the depletion of intracellular calcium stores required for photoexcitation (Hardie and Minke, 1995; Minke and Selinger, 1996). In this model, light-induced release of calcium from internal stores would activate TRP, enabling refilling of the stores and continued excitation. Store-operated calcium influx (also named capacitative calcium entry) is a process in which store depletion gates calcium entry (Putney and Bird, 1993; Berridge, 1995; Clapham, 1995). This process is essential to maintain proper calcium homeostasis in a wide range of excitable and nonexcitable cells yet its molecular components are not understood. Based primarily on the transient light response of the *trp* mutant, Drosophila phototransduction is considered a model system for the genetic dissection of store-operated calcium influx (Hardie and Minke, 1995; Minke and Selinger, 1996). We thus sought to assess whether the transient response phenotype of the *trp* mutant is indeed the result of depletion of intracellular calcium stores.

We performed whole-cell, voltage-clamp recordings of light-induced currents in wild-type and *trp* mutant photoreceptors. We reasoned that if the transient light response of the *trp* mutant resulted from loss of internal calcium, then introduction of calcium intracellularly in *trp* photoreceptors may restore responsiveness. However, *trp* mutants in the presence of high intracellular calcium (700 nm) still display transient light responses (data not shown). Also, if light responses in the *trp* mutant terminate prematurely due to loss of calcium entry, then light responses in wild-type cells in zero extracellular calcium should likewise be transient; however, this is not the

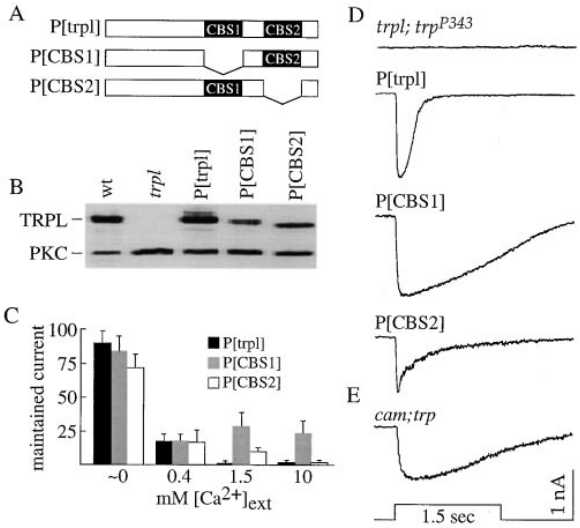

Figure 2. CAM Negatively Regulates TRPL Channel Activity

(A) The diagrams depict deletion constructs of the CAM binding sites in the TRPL ion channel. P[trpl] refers to a wild-type *trpl* cDNA, P[CBS1] contains the *trpl* cDNA with a deletion of the first CAM binding site of TRPL (aa 710–728), and P[CBS2] contains the *trpl* cDNA with a deletion of the second CAM binding site of TRPL (aa 853–895) (Warr and Kelly, 1996).

(B) Shown is a Western blot demonstrating normal levels of TRPL protein in the different transgenic flies. All studies were carried out in the *trpl; trp*^{P343} double mutant background. The blot was probed with antibodies to TRPL (Niemeyer et al., 1996). We also used antibodies to an eye-specific PKC (Smith et al., 1991) to control for sample loading errors. Each lane contains protein extracts from two fly heads. Protein extracts were prepared by sonication in 20 μl of 100 mM Tris (pH. 6.8), 3% SDS, 0.7 M β-mercaptoethanol, 10% glycerol and electrophoresed on a 10% SDS-PAGE gel (Stamnes et al., 1991).

(C) Calcium dependency of response termination in P[CBS] transgenic flies. The maintained current (defined as the percentage of the current amplitude remaining at the end of the light pulse over the peak amplitude) is plotted as a function of extracellular calcium concentration. Responses in P[CBS1] and P[CBS2] flies are far less sensitive to calcium-dependent feedback as compared to P[trpl] controls. Each bar represent the mean (±SE) from 5–10 cells from 5–10 flies. Light stimulation was a 1.5 s pulse of 580 nm light (log [I] = −1). Similar results were obtained with independent transformed lines.

(D and E) Examples of responses to pulses of light for *trpl; trp*^{P343} and for P[trpl], P[CBS1], and P[CBS2] flies in the *trpl; trp*^{P343} double mutant background (D). Light stimulation was a 1.5 s pulse of 580 nm light (log (I) = −1) and the bath solution contained 1.5 mM calcium. Under these conditions, P[trpl] controls and *trp* photoreceptors produce transient light responses (see also Figure 1A). In contrast, loss of the TRPL CAM binding sites results in light responses with prolonged currents. As expected, prolonged, intense stimulation (10 s; log [I] = −1) of P[CBS1] mutants and *trp* in zero extracellular calcium still fails to generate transient responses (data not shown). The light response in *trp* mutants also lacking CAM (*cam; trp*) is no longer transient (E). As in (D), light stimulation was a 1.5 s pulse of 580 nm light (log [I] = −1) and bath solution contained 1.5 mM calcium. Scale bars are for (D) and (E).

case (Figure 1; see also Experimental Procedures). Finally, if the transient response of *trp* is due to calcium depletion, then in the absence of extracellular calcium, responses in *trp* mutants should terminate more rapidly. In contrast, *trp* photoreceptors in zero extracellular calcium sustain robust light-induced currents (Figure 1). Taken together, these results demonstrate that the transient response of the *trp* mutant cannot be simply explained as a loss of excitation due to loss of calcium entry. Instead, the finding that *trp* mutants can maintain responsiveness in the absence of calcium suggests that there is calcium-dependent inactivation of light-induced currents in the *trp* mutant.

The light-activated conductance is composed of TRPL and TRP ion channels. In mutants lacking the TRPL and TRP channels (*trpl; trp*^{P343}), there are no light-induced currents (Figure 2D). (We previously reported that *trpl; trp*^{301} double mutants show a small light-activated conductance [Niemeyer et al., 1996]. This residual current is completely eliminated by replacing the *trp*^{301} allele with the stronger *trp*^{P343} allele). In *trp* mutants, the light-induced current is carried only by TRPL (Niemeyer et al., 1996; Hardie et al., 1997). Therefore, the transient response of *trp* reflects TRPL channel function. TRPL contains two calmodulin binding sites in its carboxyl terminus (Warr and Kelly, 1996), suggesting that it is regulated by calcium/calmodulin. Given that calcium is required for the premature termination of currents in *trp*, we hypothesized that calcium/CAM-dependent inactivation of the TRPL channel may underlie the transient response of the *trp* mutant. To examine this, we generated transgenic animals expressing TRPL channels lacking CAM binding sites 1 (P[CBS1]; aa 710–728) or 2 (P[CBS2]; aa 853–895). We also generated control animals expressing the wild-type *trpl* transgene (P[trpl]) (Figure 2A). CBS1 is a typical CAM binding site, displaying strong calcium-dependent CAM binding, while CBS2 is a novel site with calcium-independent CAM binding properties (Warr and Kelly, 1996). Each construct was placed under the control of the *trp* promoter (Montell et al., 1985; Montell and Rubin, 1989) and then introduced into hosts by P element–mediated germ line transformation (Karess and Rubin, 1984). To evaluate the function of the mutant channels in the absence of endogenous light-activated channels, all constructs were assayed in the *trpl; trp*^{P343} double mutant background. Figure 2B shows immunoblots of extracts from P[trpl], P[CBS1], and P[CBS2] flies in the *trpl; trp*^{P343} background, demonstrating the normal expression of TRPL in the P[trpl] control and showing that loss of the CAM binding sites does not affect TRPL expression in the P[CBS1] and P[CBS2] flies.

Figure 2D shows sample traces of currents evoked by a long pulse of bright light under standard recording conditions for P[trpl], P[CBS1], and P[CBS2] photoreceptors in the *trpl; trp*^{P343} background. In P[trpl] controls (and in *trp* photoreceptors), responses to long pulses of light are transient, decaying to baseline in less than 500 ms. In contrast, P[CBS1] (and P[CBS2]) cells maintain robust inward currents for the duration of the stimulus. The finding that deletion of either CAM binding site of TRPL results in a prolonged current suggests that CAM binding functions to inactivate TRPL. We also assayed the calcium dependency of light responses for each transgenic line and found that P[CBS1] responses have lost most of their calcium dependency and P[CBS2] responses have a significant shift in their calcium dependence (Figure 2C).

Taken together, our results are inconsistent with the

view that the *trp* transient phenotype results from the loss of a capacitative calcium entry channel, leading to depletion of intracellular calcium stores. Instead, these results can be explained by assuming that calcium/calmodulin binding to TRPL leads to the calcium/calmodulin-dependent inactivation of the TRPL channel and the transient light response of *trp* mutants.

A Viable Mutant in CAM

Because deletion of the CAM binding sites of TRPL affects response termination in *trp* mutants, one would predict that a *Cam; trp* double mutant should not display transient light responses. To determine how CAM participates in phototransduction, to confirm that CAM regulates the TRPL ion channel, and to define other molecular targets of CAM in the visual cascade, we set out to characterize Drosophila *Cam* mutants. In Drosophila, there is a single *Cam* gene (Yamanaka et al., 1987; Doyle et al., 1990) and null mutations are larval lethal (Heiman et al., 1996). To examine phototransduction in the absence of CAM, we generated *Cam* null photoreceptors by using the FLP-FRT system to induce mitotic recombination in *Cam* null heterozygotes (Xu and Rubin, 1993). However, photoreceptor cells lacking CAM displayed severe developmental and morphological defects, precluding their physiological characterization (data not shown).

We reasoned that if CAM levels are critical for phototransduction, then viable hypomorphic alleles of *Cam* may provide an avenue to study CAM function. We used a hypomorphic *Cam* mutant (Cam^{352}) generated by imprecise excision of a P element inserted 34 bp upstream of the *Cam* transcription start site (Heiman et al., 1996). Excision of the P element deleted approximately 4.5 kb of DNA in the promoter region of *Cam* (data not shown). Disruption of this region results in a severe reduction in CAM levels. To further reduce the amount of CAM, we performed all analyses on flies carrying one copy of the Cam^{352} mutant allele and one copy of the Cam^{n339} null allele (Heiman et al., 1996) (hereafter referred to as *cam*). Figure 3A shows a Western blot demonstrating the reduced expression of CAM in the *cam* hypomorph; these mutants express approximately 10% levels of wild-type CAM. The *cam* hypomorphs do not eclose from their pupal cases and, if assisted in eclosion, they show severe uncoordination leading to low viability. However, *cam* photoreceptor cells are not different from wild-type cells by ultrastructural examination (data not shown).

CAM Is Essential for Calcium-Dependent Deactivation of Light Responses

We performed whole cell, voltage-clamp recordings of light-induced currents in wild-type and *cam* mutant photoreceptor cells. Figure 3B shows sample traces of light responses in a wild-type cell and in a *cam* mutant cell at different light intensities. The *cam* light responses have dramatic defects in deactivation kinetics, displaying greatly prolonged deactivation times (Figure 3B and Table 1). Importantly, the defect of *cam* responses is limited to the deactivation phase of the light response. The latencies and rise times of light-induced currents in *cam* cells and the sensitivity of *cam* photoreceptors to light are not different from wild-type cells (Table 1).

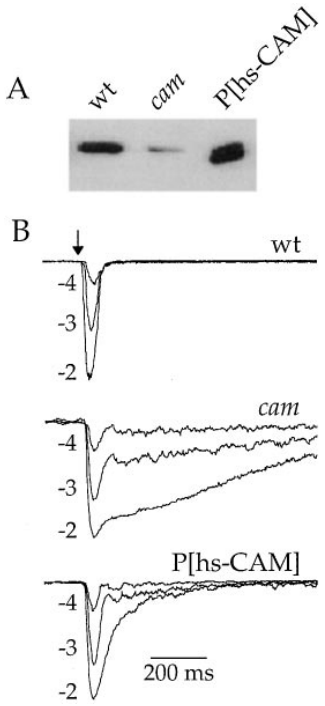

Figure 3. *cam* Mutants Have Severe Defects in Deactivation

(A) CAM expression was examined in wild-type, *cam,* and transgenic flies expressing the *Cam* gene under the control of a heat shock inducible promoter (P[hs-CAM]). The immunoblot demonstrates reduced expression of CAM in *cam* mutant flies (less than 10% by scanning densitometry measurements). The right lane shows that heat shock induction of the *Cam* transgene results in increased expression of CAM in the *cam* mutants. Expression in the transgenic line was induced by the following heat shock regime: a cycle of 1 hr at 37°C, 30 min at 22°C for a total of 4 cycles. After the fourth cycle, flies were left to recover at 22°C for 2 additional hr and then assayed. Each lane contains protein extracts from two fly heads. Control blotting with antibodies to eye-PKC showed that identical amounts were loaded in each lane (data not shown).

(B) Representative traces of light-activated currents from wild-type controls, *cam* mutants, and P[hs-CAM] in a *cam* background. Cells were stimulated with 10 ms flashes of increasing light intensity (580 nm). Numbers refer to the log order of light intensity associated with that light response (e.g., −4 is 10 times less light than −3). Note the dramatic deactivation defect of the *cam* mutant and the rescue of this defect by expression of the *Cam* transgene. Responses are normalized to the peak amplitude at log (I) = −2. Similar responses to 10 ms flashes of 580 nm light were obtained from 6–8 cells of each genotype. See Table 1 for quantitation of response parameters.

The deactivation defect of *cam* hypomorphs results from the reduction in CAM levels because increasing CAM levels in the mutant line rescues the defect. Introduction of the wild-type *Cam* gene under the control of a heat shock promoter leads to heat shock–inducible partial rescue of the *cam* deactivation defect (Figure 3B and Table 1). Expectedly, the degree of rescue correlates with the number of heat shocks given and the amount of CAM expressed (data not shown). These results demonstrate that reduced levels of CAM are limiting for rapid termination of this cascade.

To examine if CAM mediates the regulatory effects of calcium on phototransduction, we analyzed light responses of wild-type and *cam* cells in various extracellular calcium concentrations. In the absence of extracellular calcium, *cam* and wild-type responses are not

Table 1. Response Kinetics of *cam* Photoreceptors

Genotype	# Cells	Amplitude (nA)	Latency (ms)	Rise Time (ms)	Decay Time (ms)
Wild-Type	6	2.3 ± 0.6	25 ± 1	17 ± 1	40 ± 2
cam	7	2.1 ± 0.6	29 ± 3	20 ± 2	590 ± 170
cam, P[hs-cam]	8	2.2 ± 0.6	25 ± 2	22 ± 3	200 ± 20
cam, *trpl*	6	1.7 ± 0.3	34 ± 6	22 ± 7	440 ± 80

Mean values (±SE) for amplitude, latency (defined as the time elapsed from the onset of the light stimulus to the beginning of the response), rise time (measured as the time between 10% and 90% of the peak amplitude), and decay time (measured as the time between 10% and 90% deactivation). Light-induced currents were evoked by a 10 ms flash of log (I) = −3. Multiple cells representing more than 5 different flies were studied for each genotype.

significantly different from each other, demonstrating that calcium entry is required to reveal the *cam* phenotype and highlighting the absolute requirement for calcium for rapid deactivation of the phototransduction cascade (Figures 4A and 4B). As expected, the deactivation times of wild-type light responses decrease as extracellular calcium is increased (Figures 4C and 4D). Remarkably, *cam* mutant responses have lost much of this calcium dependency and display similar deactivation kinetics in 0.4, 1.5, and 10 mM external calcium (Figures 4C and 4D). These results indicate that CAM is one of the primary targets for the light-induced calcium entry and that CAM functions in a regulatory loop in which activation of the visual cascade leads to the entry of extracellular calcium, which in turn triggers deactivation by activating CAM.

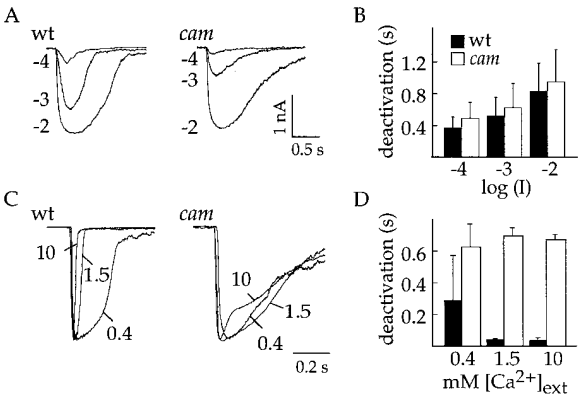

Figure 4. Calcium-Dependent Negative Regulation Is Mediated by CAM

(A) Light-activated currents of wild-type controls and *cam* mutants in the absence of extracellular calcium. Cells were stimulated with 10 ms flashes of increasing light intensity (580 nm). Numbers refer to the log order of light intensity associated with that response.

(B) Mean deactivation times (±SE) of light responses in the absence of extracellular calcium for wt (n = 6) and *cam* (n = 8) photoreceptors at different light intensities. In the absence of extracellular calcium, deactivation times of responses in wt and *cam* are not significantly different.

(C and D) Superimposed current traces in 0.4, 1.5, and 10 mM extracellular calcium for wild-type and *cam* photoreceptors (D). In wild-type cells, deactivation times of responses decrease as calcium concentration is increased. In contrast, light responses in *cam* cells show similar deactivation kinetics at all calcium concentrations. Cells were stimulated with 10 ms flashes of 580 nm light (log [I] = −1). Response traces are normalized to the peak amplitude. (D) Bar graph showing quantitation of recordings similar to (C). Each bar (±SE) represents the average response of 5–10 cells from 5–10 flies.

CAM Regulates Multiple Components of Phototransduction

Since the transient phenotype of the *trp* mutant results from CAM regulation of the TRPL ion channel, responses to pulses of light should not be transient in *cam; trp* double mutants and may resemble responses of the transformants with TRPL lacking CAM binding sites. Indeed, Figure 2E shows that *cam* mutants lacking TRP ion channels do not have transient light responses. This confirms and extends our demonstration that calmodulin-dependent inactivation of TRPL can account for the transient response of the *trp* mutant.

If TRPL is the only target of CAM and if inappropriate activity of TRPL fully accounts for the deactivation defect of the *cam* mutant, then light responses in *cam* mutants lacking TRPL ion channels should display normal deactivation kinetics. Table 1 shows that the deactivation times of light responses in *cam, trpl* double mutants are still defective, demonstrating that additional phototransduction components are regulated by CAM.

Quantum Bumps Are Continually Produced in the *cam* Mutant

In order to define the nature of the additional component(s) regulated by CAM and to determine the underlying basis for the deactivation defect of *cam* mutants, we analyzed quantum bumps in *cam* photoreceptors. In wild-type photoreceptors, single photons give rise to unitary responses known as quantum bumps (Yeandle, 1957; Baylor et al., 1979). A quantum bump results from the activation of a single rhodopsin molecule and reflects the amplification of the entire signaling pathway, culminating in the opening of the light-activated ion channels. In Drosophila, mutations that affect transduction steps upstream of PLC activation change quantum bump frequency but do not affect quantum bump size or shape (Scott et al., 1995; K. S. and C. S. Z., unpublished data). This argues that there is very little amplification in the initial steps of the phototransduction cascade and suggests that activation of one rhodopsin results in the activation of only a few G proteins and PLCs. In contrast, mutations that reduce the number of light-activated channels change quantum bump size, demonstrating that many ion channels are opened in response to a photon of light (Niemeyer et al., 1996). Thus, the distal events of phototransduction must provide most of the amplification for this signaling cascade. We reasoned that if CAM is important for the regulation of signaling components upstream of PLC activation, then the frequency of quantum bumps should be affected in

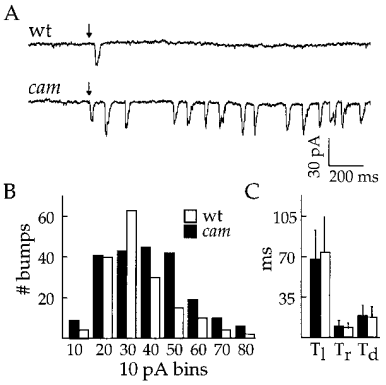

Figure 5. A Single Photon Produces a Train of Quantum Bumps in *cam* Mutants

(A) Sample traces of quantum bumps in wild-type and *cam* mutants induced by a 10 ms flash of 580 nm light of log (I) = −6. In *cam* photoreceptors, activation of one rhodopsin (arrow) generates a train of many quantum bumps.

(B) Amplitude histograms demonstrate that the mean bump amplitude is not notably different in *cam* cells. Response amplitudes were measured for 200 *cam* and 200 wild-type quantum bumps. The probability of a quantum bump occurring (Ps) was 0.55 for wild-type and 0.28 for *cam* (assuming a Poisson distribution of events and solving for Ps = −1 [trials with zero quantum bumps/total trials]) (Baylor et al., 1979).

(C) Kinetics of quantum bumps. Shown are bar graphs comparing latency (Tl), rise time (Tr), and deactivation time (Td) of wild-type and *cam* quantum bumps. Latency was measured as the time from the onset of stimulus to the initiation of the current. Rise time was measured as the time between 10% and 90% peak response amplitude. Deactivation time was measured as the time between 90% and 10% decay of peak amplitude. There are no significant differences between wild-type and *cam* responses.

the *cam* mutant. On the other hand, if CAM serves to deactivate distal components of signaling (i.e., the light-activated ion channels), then the size and shape of quantum bumps should be altered.

For generation of quantum bumps, photoreceptors were stimulated with 10 ms flashes of low intensity light such that only a fraction of the flashes elicited responses (Baylor et al., 1979). Both wild-type and *cam* mutant cells show similar sensitivity to light (data not shown). Remarkably, *cam* photoreceptors display a dramatic increase in the number of quantum bumps generated from a single flash of light (Figure 5A). In wild-type cells, activation of one rhodopsin produces a quantum bump. In *cam* mutant cells, activation of one rhodopsin produces many quantum bumps, resulting in a quantal train of bumps. Despite the difference in the number of quantum bumps generated from a single photon of light, the amplitudes and kinetics of individual quantum bumps in wild-type and *cam* cells are similar to each other (Figures 5B and 5C). These results demonstrate several important aspects of calmodulin function in phototransduction. First, because quantum bumps are continuously produced in the *cam* mutant, CAM must be required to turn off a signaling component(s) that is activated in response to one photon of light. Thus, feedback regulation of phototransduction must operate even at the lowest levels of light detection, and calmodulin must be exquisitely sensitive to small localized changes in calcium. Second, because *cam* mutant responses are trains of discrete quantum bumps rather than a continued response, a refractory period or a transient inactivation is still present in *cam*. This demonstrates that there are CAM-independent mechanisms of deactivation. It also shows that a signaling component(s) is inappropriately recycling between the active and the inactive state in the absence of CAM. Finally, because the shape of the individual bumps in *cam* is not notably different than wild type, the primary site of CAM action at the level of single photon responses must be upstream of quantum bump shape determinants and therefore upstream of the light-activated ion channels.

CAM-Dependent Deactivation of Rhodopsin

To localize the requirement for CAM in the deactivation of single photon responses, we performed a genetic epistasis experiment. Previously, we demonstrated that mutants with reduced levels of the G protein involved in phototransduction ($G\alpha q^1$ mutants) display quantum bumps with a very low probability of occurring and very long latencies (Scott et al., 1995). This showed that the in vivo levels of G protein do not regulate the amplitude of single photon responses and that the G protein functions primarily as an on/off switch reporting rhodopsin activity to downstream components. If CAM is required for the deactivation of a signaling component upstream of the G protein (i.e., rhodopsin), then a reduction in the levels of G protein should prevent or reduce the quantal trains in a *cam*, $G\alpha q^1$ double mutant because each bump in a train would require the activation of additional G proteins. On the other hand, if CAM is important for the deactivation of a component downstream of the G protein, then a reduction in the levels of G protein should not affect the production of multiple quantum bumps in a *cam*, $G\alpha q^1$ double mutant, only the latency to the first bump. Figure 6A shows sample traces of quantum bumps in a *cam*, $G\alpha q^1$ double mutant, demonstrating that quantal trains of bumps are no longer produced. Thus, the continuous activity of the *cam* mutant requires continual signaling through the G protein, indicating that the component that is inappropriately activated in the absence of CAM is upstream of the G protein (i.e., rhodopsin).

In Drosophila photoreceptors, the catalytic lifetime of activated rhodopsin is determined by arrestin binding (Ranganathan and Stevens, 1995). Visual arrestins regulate rhodopsin function by binding to the receptor and uncoupling it from the downstream G protein. A logical way for CAM to regulate rhodopsin function would be to modulate its interaction with arrestin. In fact, the Drosophila photoreceptor-specific Arr1 and Arr2 proteins are phosphorylated in a light- and CAM-dependent manner (Byk et al., 1993; Matsumoto et al., 1994). Since Arr2 is the most abundant visual arrestin, we assayed its phosphorylation state in *cam* mutants. Figure 6C demonstrates that Arr2 is no longer phosphorylated in the *cam* background. To further validate the notion that the lack of arrestin regulation could be responsible for the multiple bumps in *cam* photoreceptors, we examined quantum bumps in arrestin mutants. Since these cells have a complete loss of arrestin function, we expected that an arrestin mutant would represent a more

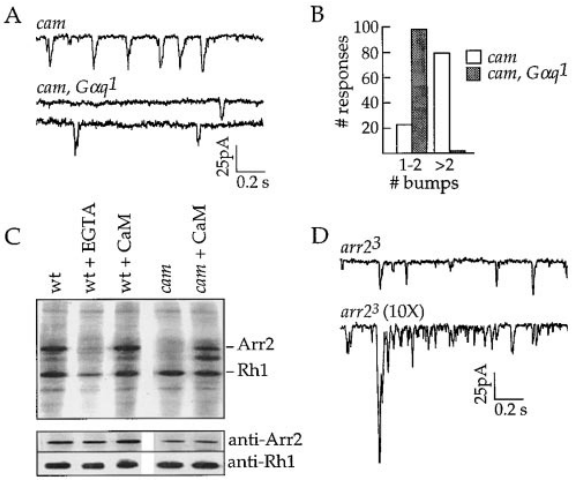

Figure 6. CAM Acts Upstream of the G Protein

(A) Quantum bumps in *cam* and *cam, Gαq¹* mutant photoreceptors. Quantum bumps were stimulated by a 10 ms flash of 580 nm light of log (I) = −6 for *cam* and log (I) = −3 for *cam, Gαq¹* given at the onset of the trace.

(B) The number of quantum bumps per response is shown for *cam* and *cam, Gαq*, demonstrating that trains of quantum bumps are not produced in the *cam, Gαq* mutant (N = 100 bumps for each genotype).

(C) Phosphorylated proteins from retinal membranes of wild-type and *cam* mutants are shown in the upper panel. Arrestin2 (Arr2) and rhodopsin (Rh1) are the major phosphoproteins in the retina. Note that Arr2 is phosphorylated in a calcium-dependent manner in wild-type extracts and is not phosphorylated in *cam* mutant extracts. Introduction of purified bovine CAM restores arrestin phoshorylation in *cam* mutants. The lower panel shows the same blot probed with antibodies to Arr2 (Dolph et. al., 1993) and Rh1 to demonstrate equivalent protein levels in each lane.

(D) Samples of quantum bumps in wt and *arr2³* mutants stimulated by a 10 ms flash of 580 nm light of log (I) = −6, or with ten times (10×) more light (log [I] = −5) for *arr2³*, at time = 200 ms. Mutants lacking Arr2 display many quantum bumps in the absence of light stimulation and an enhanced number upon light stimulation.

extreme phenotype than the *cam* mutant. Indeed, Figure 6D shows that *arr2³* photoreceptors display continuous bump activity even in the absence of light stimulation and that macroscopic currents show continued light-induced bumps even after termination of the stimulus. This inappropriate transduction activity is the result of loss-of-arrestin function, since it is absent in control flies, and represents continued rhodopsin activity since it is suppressed in mutants lacking rhodopsin (Dolph et al., 1993; data not shown). Taken together, these results validate rhodopsin as a critical target of feedback regulation and indicate that defects in rhodopsin shutoff underlie the bump phenotype of *cam* mutants.

Concluding Remarks

In this manuscript, we describe the characterization of a novel *Cam* mutant in a complex multicellular organism and its role in a well-defined signaling cascade. We use a viable hypomorphic allele expressing ∼10% of normal CAM levels, a heat-inducible *cam* transgene, and detailed electrophysiology to show that the in vivo levels of CAM regulate photoreceptor cell deactivation. We also mapped the sites of action of CAM to specific transduction proteins.

We demonstrated that CAM modulates the function of TRPL light-activated channels in vivo. Because the light-activated conductance is composed of TRP and TRPL channels and because TRPL represents only a small fraction of the total current (Niemeyer et al., 1996), *trpl* transgenes lacking CAM binding sites have no phenotype in a wild-type background. However, the TRPL channel mutants should reveal their phenotype in a *trpl; trp* double mutant background. Indeed, we showed that calcium/calmodulin regulates deactivation of the TRPL ion channel and that this can account for the transient light response of the *trp* mutant. During the past several years, the TRP channel has become a favorite model for the study of capacitative calcium entry. This was largely based on the observations that phototransduction in Drosophila requires intracellular calcium (Hardie et al., 1993), that TRP is a calcium-permeable ion channel (Hardie and Minke, 1992), and that *trp* mutants display only a transient response to prolonged, intense illumination (Cosens and Manning, 1969). This phenotype was interpreted as resulting from the stimulus-dependent depletion of internal stores and the loss of the primary calcium entry pathway (Hardie and Minke, 1992, 1995; Minke and Selinger, 1996). Recently, TRP homologs have been found in a variety of species and hypothesized to function as store-operated channels (Petersen et al., 1995; Wes et al., 1995; Zhu et al., 1996; Zitt et al., 1996). Our results do not support an internal store depletion model of excitation based on the *trp* phenotype or a requirement for TRP channels in the refilling of the internal stores. Future experiments may help determine whether the internal store depletion model plays any role in this pathway (see Acharya et al., 1997, for additional evidence against this model).

We also showed that CAM is required for rhodopsin deactivation. Having activity-dependent feedback regulation at the first step of this signaling cascade, prior to any amplification, makes functional sense. On the one hand, the requirement for calcium entry and CAM activation ensures that deactivation mechanisms are only triggered after successful excitation (i.e., opening of the light-activated channels). On the other hand, modulation at the first step of the cascade (i.e., receptor level) minimizes the number of signaling molecules that need be regulated for immediate feedback control of the light response. Interestingly, Lagnado and Baylor, (1994), used a novel truncated rod preparation to show that the active lifetime of vertebrate rhodopsin could be regulated in real time by calcium. Our studies also show that the calcium-dependent feedback of Drosophila rhodopsin operates even at the lowest light levels (i.e., quantum bumps), demonstrating that the phototransduction signaling cascade is exquisitely sensitive to small localized changes in calcium.

A number of studies have previously suggested that CAM may be involved in the gating of the light-activated channels (Warr and Kelly, 1996) or the excitatory phase of the phototransduction cascade (Arnon et al., 1997). Although it is technically impossible to generate complete CAM nulls to rigorously eliminate this possibility, our studies with severe hypomorphic alleles failed to

reveal a role for CAM in this aspect of the phototransduction cycle. Also, contary to recent reports suggesting TRPL must form obligatory heteromultimers with the TRP ion channel (Xu et al., 1997), we demonstrated that wild-type and mutant TRPL channels function in vivo in the absence of any *trp* gene product (see also Niemeyer et al., 1996 and Tsunoda et al., 1997).

Finally, our finding that CAM regulates multiple steps within the same signaling cascade and the demonstration that CAM functions as a primary target of extracellular calcium entry, highlight a hierarchical network of signal regulation and provide an experimental paradigm for the dissection of the role of calcium in a G protein–coupled receptor signaling pathway.

Experimental Procedures

Electrophysiological Recordings

Photoreceptors were isolated from adult flies (<6 hr after eclosion) and whole-cell, voltage-clamp recordings were performed as previously described (Ranganathan et al., 1991). The bath solution contained 124 mM NaCl, 4 mM KCl, 10 mM HEPES, 5 mM Proline, 25 mM Sucrose (pH 7.15). Unless otherwise stated, the bath solution also contained 1.5 mM $CaCl_2$. For experiments in zero extracellular calcium, extra care was taken to insure that cells were analyzed within a few minutes. Under these conditions, wild-type and mutant cells do not run down. The pipette solution contained 95 mM potassium gluconate, 40 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$, 0.2 mM EGTA (pH 7.15).

Photoreceptors were clamped at a holding potential of −70 mV, and series resistances were compensated at ∼80%. In all experiments, light was filtered through a bandpass filter ($\lambda = 580 \pm 10$ nm), through neutral density filters, and focused onto the photoreceptor cells through a 0.5 numerical aperture, 40× objective. Stimulation was by means of a 75 W Xenon source; unattenuated output at the stage was 10 mW for white light.

For quantum bump analysis, photoreceptors were clamped at a holding potential of −70 mV. Signals were sampled at 1 KHz, then filtered at 500 Hz.

DNA Constructs and Transgenic Flies

A 3500 bp DNA fragment containing the entire coding region of *trpl* (Phillips et al., 1992) was used as a template for oligonucleotide site-directed mutagenesis of the CAM binding sites in TRPL (Maniatis et al., 1982). For P[CBS1], we deleted residues 710–728 and for P[CBS2], residues 853–895 were deleted (Warr and Kelly, 1996) (amino acid numbers are according to Phillips et al., 1992). All PCR products and reconstructed genes were sequenced in their entirety. The mutated *trpl* genes were placed under the regulatory control of the *trp* promoter (Montell et al., 1985; Montell and Rubin, 1989) and subcloned into the pCasper-4 Drosophila transformation vector. P element–mediated germline transformations and all subsequent fly manipulations were performed using standard techniques (Karess and Rubin, 1984).

Generation of *cam* Mutants and Fly Stocks

A homozygous viable P-element (w^+) insertion, located at position 34 bp relative to the calmodulin transcription start site, was mobilized by hybrid dysgenesis. Individual chromosomes showing a loss of the w^+ marker were assayed for homozygous adult lethality and screened by genomic Southern blots to identify deletions flanking the insertion site (Heiman et al., 1996).

All experiments with the *trp* mutant used the $w;trp^{P343}$ allele. Control flies were either *W1118* or *yw* (for *cam* experiments). *cam* hypomorphs were generated by crossing $yw;Cam^{352}/Cyo(y+)$ males to $yw;Cam^{339}/Cyo(y+)$ virgins and selecting $yw;Cam^{352}$/Cam339 larvae.

Phosphorylation of Retinal Proteins

Retinal membranes were prepared as previously described (Byk et al., 1993). Membranes were illuminated with white light for 15 min in the presence of 1 μCi [γ^{32}P]ATP, 10 mM MOPS, 3 mM $MgCl_2$, 120 mM KCl, 1 mM DTT, 0.1 mM $CaCl_2$ with leupeptin at 10 μg/ml and pepstatin A at 1 μg/ml (pH 7.0). Immediately after incubation, proteins were electrophoresed on a 10% SDS-PAGE gel (30 retinas/lane). When indicated, 1 mM EGTA was substituted for $CaCl_2$ or 2 μM bovine CAM (Calbiochem) was added.

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