Calmodulin Regulation of Calcium Stores in Phototransduction of *Drosophila*

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Phototransduction in *Drosophila* occurs through the ubiquitous phosphoinositide-mediated signal transduction system. Major unresolved questions in this pathway are the identity and role of the internal calcium stores in light excitation and the mechanism underlying regulation of Ca^{2+} release from internal stores. Treatment of *Drosophila* photoreceptors with ryanodine and caffeine disrupted the current induced by light, whereas subsequent application of calcium-calmodulin (Ca-CaM) rescued the inactivated photoresponse. In calcium-deprived wild-type *Drosophila* and in calmodulin-deficient transgenic flies, the current induced by light was disrupted by a specific inhibitor of Ca-CaM. Furthermore, inhibition of Ca-CaM revealed light-induced release of calcium from intracellular stores. It appears that functional ryanodine-sensitive stores are essential for the photoresponse. Moreover, calcium release from these stores appears to be a component of *Drosophila* phototransduction, and Ca-CaM regulates this process.

In muscles, neurons, and various nonexcitable cells, the endoplasmic reticulum is the main Ca^{2+} store that releases Ca^{2+} through the inositol 1,4,5-trisphosphate ($InsP_3$) receptor or the ryanodine receptor (RyR) (*1–3*). Ryanodine (*1*) and caffeine (*2*) release Ca^{2+} through the RyR, which is negatively regulated by Ca-CaM (*2*, *4*, *5*). The RyR also responds to an increase in the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$), thereby mediating Ca^{2+}-induced Ca^{2+} release (*1–6*).

A major unresolved question of calcium signaling in vivo is the regulation of Ca^{2+} release from internal stores (*2*, *4*). An approach that may provide an insight into this complex signaling is the genetic dissection of *Drosophila* phototransduction that is mediated by phosphoinositide signaling (*7–9*). Because phospholipase C is required for *Drosophila* phototransduction (*9*, *10*), $InsP_3$ and Ca^{2+} were considered as putative messengers of *Drosophila* light excitation. However, an excitatory action of Ca^{2+} on *Drosophila* photoreceptors has not been demonstrated (*11*), and fluorimetric measurements of $[Ca^{2+}]_i$ failed to detect a light-induced Ca^{2+} release from internal stores (*12–14*). These studies indicated that Ca^{2+} release from internal stores was unlikely to mediate activation by light of *Drosophila* photoreceptors (*13*, *14*). Ryanodine- and caffeine-sensitive Ca^{2+} stores and a Ca^{2+}-induced Ca^{2+}-release mechanism exist in photoreceptors of the honeybee drone, and caffeine affects facilitation and adaptation of the photoreceptor cells (*15*). *Drosophila* photoreceptors contain abundant amounts of CaM and have a specific protein, NINAC (neither inactivation nor afterpotential C), that stockpiles large amounts of CaM in the cell (*16*). The cloned RyR of *Drosophila* contains CaM-binding domains (*17*). However, the function of CaM in *Drosophila* phototransduction is still unknown. Thus, the major unresolved questions of *Drosophila* phototransduction are the function and identity of the internal Ca^{2+} stores and whether CaM regulation of Ca^{2+} release from internal stores is essential for excitation by light.

To address these questions, we used whole-cell voltage-clamp recordings from isolated *Drosophila* ommatidia (*18*) and applied chemical agents that release Ca^{2+} from internal stores. Application of ryanodine, caffeine, or both, either to the bath or through the recording pipette to *Drosophila* photoreceptors abolished the light-induced current (LIC) (Fig. 1A) (*19*). This abolishment was accelerated by illumination (n = 8). Control photoreceptors bathed in normal solution or in solution that included 0.1% dimethyl sulfoxide (DMSO) did not reveal any disruption of the photoresponse (Fig. 1A).

When inactivated photoreceptors treated with ryanodine and caffeine were internally dialyzed with Ca-CaM (5 μM CaM, 10 μM Ca^{2+}), a normal light response was restored within 1 min (*19*, *20*) (Fig. 1A). The complete restoration of the LIC by Ca-CaM in ryanodine-treated cells was also obtained at a lower dose (0.2 μM) of Ca-CaM (n = 4) (*21*) or when the ryanodine concentration was increased to 100 μM

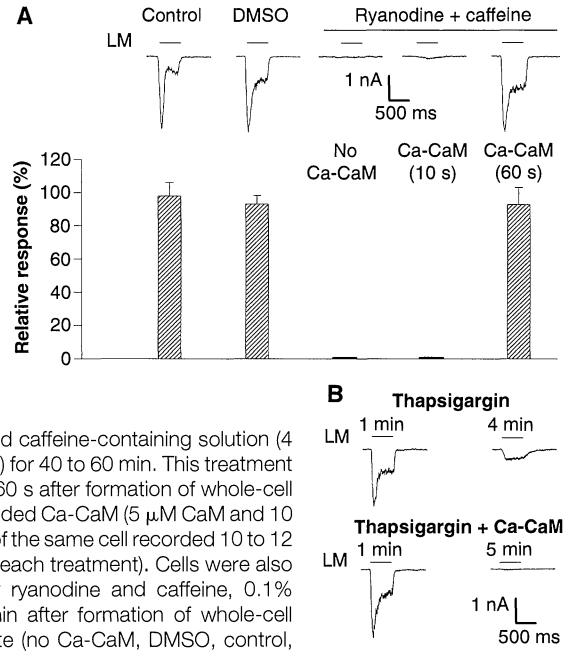

Fig. 1. Ca-CaM restores the light response after abolishment with ryanodine and caffeine (**A**) but not after abolishment with thapsigargin (**B**). In Figs. 1 to 3, whole-cell voltage-clamped inward current measurements are shown in response to constant orange (OG-590 Schott, 500 ms) light pulses (the thin line above or below the traces indicates the time when light was applied, LM). Light was attenuated by 1 log unit at holding potentials of −60 mV. All the experiments were done in normal Ringer solution except where indicated. The time from the formation of the whole-cell recordings is indicated. (A) Cells were incubated in a ryanodine- and caffeine-containing solution (4 and 10 μM, respectively, in 0.1% DMSO) for 40 to 60 min. This treatment eliminated the LIC. The peak LIC 10 or 60 s after formation of whole-cell recording, with pipette solution that included Ca-CaM (5 μM CaM and 10 μM Ca^{2+}), was divided by the peak LIC of the same cell recorded 10 to 12 min later (Ca-CaM 10 s, 60 s; n = 5 for each treatment). Cells were also treated with bath application of either ryanodine and caffeine, 0.1% DMSO, or normal Ringer solution 1 min after formation of whole-cell recording without Ca-CaM in the pipette (no Ca-CaM, DMSO, control, respectively). The peak LIC 10 to 12 min after application of chemicals was divided by the peak LIC before application of chemicals in the same cell. Geometrical averages of the ratios were used to summarize the data (bar graphs). The current trace above each bar graph shows an example of a response to light that was measured at the end of the chemical treatments. (**B**) (Upper trace) Internal dialysis with thapsigargin (10 μM in 0.1% DMSO). (Lower trace) Addition of Ca-CaM (5 μM CaM and 10 μM Ca^{2+}) to the pipette.

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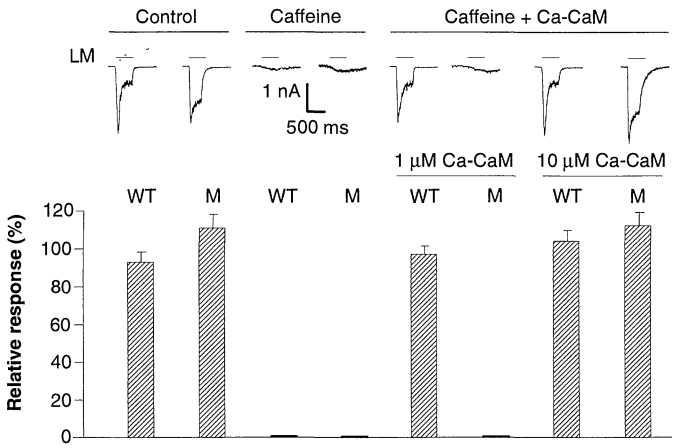
(n = 4). The effect of Ca-CaM was completely abolished by application of M5 (*19*), a specific peptide inhibitor of Ca-CaM (*22*). Inhibition of the microsomal Ca^{2+}-dependent adenosine triphosphatase by thapsigargin or cyclopiazonic acid (*23*) initially reduced the amplitude and subsequently eliminated the LIC. This abolishment, however, was not prevented by Ca-CaM (Fig. 1B) (*19*), suggesting that Ca^{2+} in stores is required for the LIC. Thus, Ca-CaM antagonizes the effects of ryanodine and caffeine but not thapsigargin.

We further examined the protective effect of Ca-CaM using flies in which the CaM concentration in the photoreceptors was reduced by expression of an altered *nina*C transgene, P[*nina*C$^{\Delta B}$] (*16*, *24–26*). In this transgene, the DNA sequence encoding the CaM binding sites of *nina*C is deleted (*16*). The LIC of P[*nina*C$^{\Delta B}$] was greater during the plateau phase and had a slower response termination than that of wild-type (WT) flies (n = 7) (*27*). Both caffeine and ryanodine blocked the LIC of P[*nina*C$^{\Delta B}$] as in WT cells (*19*). However, compared with cells from WT flies, a much larger concentration of Ca-CaM was required to rescue the blocking effect of caffeine in P[*nina*C$^{\Delta B}$] flies (Fig. 2). This result is consistent with the reduced amount of CaM in P[*nina*C$^{\Delta B}$] flies (*16*) and suggests that in WT photoreceptors endogenous Ca-CaM may participate in regulation of Ca^{2+} release from internal Ca^{2+} stores.

A critical question is whether Ca-CaM plays a regulatory role in light excitation that can be demonstrated without application of ryanodine and caffeine. We diminished the effective amount of cellular Ca-CaM by reducing either the $[Ca^{2+}]_i$ or the amount of endogenous CaM. In the presence of external Ca^{2+}, the LIC has an initial transient phase followed by a plateau phase of smaller amplitude caused by Ca^{2+} influx that mediates light adaptation (*28*). When Ca^{2+} was removed from the external medium, light adaptation was reversibly eliminated (Fig. 3A). To further reduce the effective concentration of endogenous Ca-CaM, we internally dialyzed the cells through the recording pipette with M5 and removed Ca^{2+} from the external medium. These conditions virtually abolished the LIC (*19*). Partial recovery of the photoresponse was obtained when Ca^{2+} was restored to the external medium (Fig. 3B). The blocking effect of M5 in Ca^{2+}-free medium was prevented when M5 was dialyzed together with Ca-CaM but not when M5 was applied together with free CaM (Fig. 3, C and D) (*19*) or with Ca^{2+} (*19*). Apparently, removal of Ca^{2+} from the external medium was required to make Ca-CaM a limiting factor for excitation. At normal concentrations of external Ca^{2+}, the large Ca^{2+} influx during illumination (*12–14*) and the ensuing production of Ca-CaM probably override the effect of M5.

As an alternative method to reduce the amount of Ca-CaM in the cells, we used P[*nina*C$^{\Delta B}$] flies. Application of M5 into the photoreceptors of P[*nina*C$^{\Delta B}$] flies largely inhibited the photoresponse within 4 min and virtually abolished the light response within 10 min in the presence of 1.5 mM external Ca^{2+} (Fig. 3E) (*19*). This disruption of the LIC appeared to result from blocking of Ca-CaM action because addition of Ca-CaM restored the LIC (Fig. 3F) (*19*). Application of Ca^{2+}-free CaM (Fig. 3G) (*19*) or Ca^{2+} (*19*) rather than Ca-CaM did not reduce the effect of M5. We conclude that when Ca-CaM becomes the limiting factor in P[*nina*C$^{\Delta B}$] or in Ca^{2+}-deprived WT photoreceptors, M5



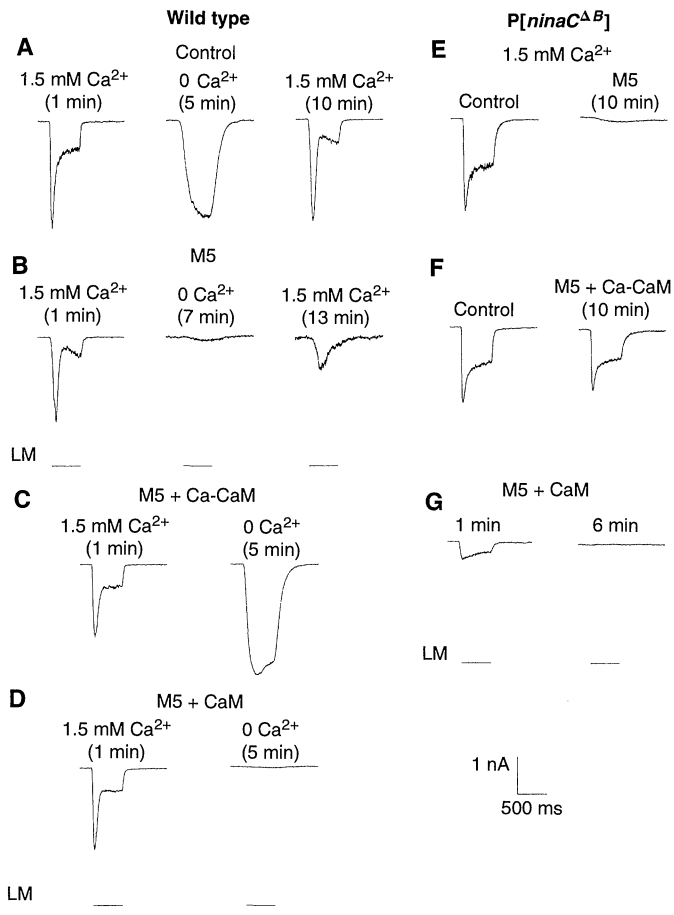


Fig. 2 (**left**). Concentration of Ca-CaM required to rescue the LIC of P[*nina*C$^{\Delta B}$] or WT cells after treatment with caffeine. The LICs of P[*nina*C$^{\Delta B}$] and WT cells were abolished by internal dialysis of caffeine (10 mM, n = 5). Ca-CaM (1 μM) restored the LIC of WT but not of P[*nina*C$^{\Delta B}$] cells (M), whereas 10 μM CaM and 20 μM Ca^{2+} rescued the LIC of both (*19*). Data presentation and averaging are as in Fig. 1A. **Fig. 3** (**right**). Ca-CaM is essential for light excitation. Each set of LIC traces was recorded from the same cell (except where indicated otherwise). Chemicals (40 μM M5, 40 μM CaM plus 80 μM Ca^{2+}, and 40 μM free CaM) were applied through the recording pipette as indicated. External $[Ca^{2+}]$ is also indicated. (**A**) The effect of removing external Ca^{2+} on the waveform of the LIC is shown. (**B**) M5 reversibly blocked the LIC of WT flies bathed in Ca^{2+}-free medium. (**C**) Ca-CaM protected the LIC from the blocking effect of M5 in Ca^{2+}-free medium. (**D**) Free CaM without added Ca^{2+} did not protect the LIC from the blocking effect of M5. The disruption of the LIC was not due to Ca^{2+} buffering by CaM because inclusion of 0.4 mM EGTA (n = 4) or 40 μM BAPTA (n = 4) without CaM did not abolish the LIC. (**E**) M5 blocked the LIC of the transgenic flies, P[*nina*C$^{\Delta B}$], at normal external $[Ca^{2+}]$. Control responses in (E) and (F) were recorded from other cells in the same retina without chemicals in the pipette. (**F**) Ca-CaM protected the LIC from the blocking effect of M5 in P[*nina*C$^{\Delta B}$]. (**G**) Free CaM did not protect the LIC from the blocking effect of M5.

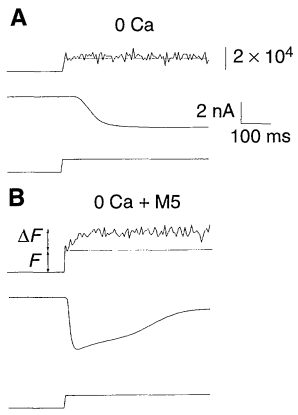


Fig. 4. A reduction in functional cellular CaM by M5 enables detection of light-induced release of Ca^{2+} from internal stores. Simultaneous recordings of LIC (middle traces) and the fluorescence of the Ca^{2+} indicator fluo-3 (100 μM, upper traces) measured in Ca^{2+}-free medium in photoreceptors of WT flies. Ca^{2+}-free (0.2 mM EGTA) solution was used to prevent influx of Ca^{2+}. The fluorescence data were sampled at 300 samples per second, and the background fluorescence was subtracted. The onset of light (indicated by a photocell current, bottom traces) elicited a LIC and excited the fluorescent dye. Because the LIC lagged behind the indicator fluorescence, the first two to four data points represent the resting $[Ca^{2+}]_i$ (F, thin line). The fluorescence increment above F reflects the rise in $[Ca^{2+}]_i$ (ΔF). $\Delta F/F$ is used to compare the rise in $[Ca^{2+}]_i$ between cells (*12*). (**A**) Control. (**B**) M5 (100 μM) was included in the recording pipette (same retina). Similar results were obtained under the same experimental paradigm in P[*ninaC*$^{\Delta B}$] cells.

inhibits light excitation by further decreasing the amount of endogenous Ca-CaM, resulting in total disruption of the photoresponse.

The disruption of the photoresponse by M5 might be explained by assuming that release of Ca^{2+} from internal stores is required for light excitation and that functional stores require a negative feedback of Ca-CaM to prevent their depletion. We made simultaneous measurements of LIC and Ca^{2+}-indicator (fluo-3) fluorescence in photoreceptors bathed in Ca^{2+}-free medium (including 0.2 mM EGTA). To estimate the release of Ca^{2+} from intracellular stores, we calculated the ratio $\Delta\bar{F}/\bar{F}$ ($\bar{F}$ is the average fluorescence reflecting the resting $[Ca^{2+}]_i$, and $\Delta\bar{F}$ is the average peak increase in fluorescence reflecting the increase in $[Ca^{2+}]_i$). Photoreceptors of both WT and P[*ninaC*$^{\Delta B}$] flies, bathed in Ca^{2+}-free medium, failed to show light-induced increase in $[Ca^{2+}]_i$ above the resting $[Ca^{2+}]_i$ ($\Delta\bar{F}/\bar{F}$ = 0.00, n = 4) (Fig. 4A) (*12–14*). However, photoreceptors that were internally dialyzed for 2 min with M5 (100 μM, same retina) did show increased $[Ca^{2+}]_i$ in response to light ($\Delta\bar{F}/\bar{F}$ = 0.79 ± 0.06 in WT and 0.61 ± 0.02 in P[*ninaC*$^{\Delta B}$]; n = 4) (Fig. 4B). The LIC in M5-dialyzed cells was larger, and reached its maximum about three times faster, than that from cells in Ca^{2+}-free medium without M5 and then decayed rapidly. The time to peak current (from light onset) was 232.2 ± 44.0 ms (n = 4) in untreated cells and 72.6 ± 26.8 ms (n = 5) in M5-treated cells, consistent with the effect of $[Ca^{2+}]_i$ on the waveform of the LIC (*28*, *29*). Thus, inhibition of endogenous Ca-CaM results in enhanced Ca^{2+} release from intracellular stores, suggesting that Ca-CaM negatively regulates the release process.

The genetic evidence that phospholipase C is required for phototransduction in *Drosophila* (*9*, *10*) and our finding that the photoresponse is disrupted both by ryanodine and caffeine raise the question as to the mechanism by which these two Ca^{2+}-release systems interact (*3*, *6*). Release of Ca^{2+} by $InsP_3$ could be the trigger for amplification and spatial spread of the Ca^{2+} signal by Ca^{2+}-induced Ca^{2+} release through the ryanodine receptor as suggested for the honeybee drone (*15*). Ca-CaM may be required to curb the release or to enhance uptake of Ca^{2+} into the internal stores during light excitation.

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18. Dissociated ommatidia from white-eyed *Drosophila* were prepared from newly emerged flies (<4 hours after eclosion), and whole-cell, patch-clamp recordings were done as described (*12*, *28*). A Zeiss Axiovert inverted microscope with a Fluar 40× objective (numerical aperture 1.3) was used together with 150-W Xenon epi-illumination and a tungsten trans-illumination sources (Schotte OG 590 filter, attenuated by 1 log unit, with effective intensity of about 2 log units below the saturating intensity for the LIC). The membrane potential of the photoreceptors was voltage-clamped at a holding potential of −60 mV. The bath solution contained 120 mM NaCl, 5 mM KCl, 10 mM *N*-tris (hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES) buffer (pH 7.15), 4 mM $MgSO_4$, and 1.5 mM $CaCl_2$ (except when Ca^{2+}-free medium was used as indicated). The whole-cell recording pipette contained 100 mM K gluconate, 10 mM TES buffer (pH 7.15), 2 mM $MgSO_4$, 4 mM Mg adenosine 5′-triphosphate, and 0.4 mM Na_2 guanosine triphosphate. When caffeine (10 mM) was used, an equivalent concentration of NaCl or K gluconate was reduced from the external or internal solution, respectively.
19. Disruption of the LIC is defined as a reduction, to <1%, of the geometrical average of the ratios between the peak LIC 10 to 12 min after drug application (40 to 60 min for ryanodine) and the peak LIC before drug application. A restored LIC was obtained when this ratio was not statistically different from 100% (paired t test, $P > 0.05$; n = 4 to 7 in all experiments).
20. The recovery time-course of the disrupted light response in cells dialyzed with Ca-CaM (<1 min) was dependent on the diffusion time of Ca-CaM from the recording pipette into the cell. This time course was determined by fluorescent measurements with fluorescein isothiocyanate–labeled CaM (Sigma). Ca^{2+}-free solution without Ca^{2+} buffers contains about 2 to 3 μM Ca^{2+}. Therefore, when we applied CaM, ≤ 1 μM Ca^{2+} was not added to the pipette.
21. The requirement for exogenous Ca-CaM to rescue the LIC after disruption by ryanodine and caffeine despite abundant CaM in the photoreceptors may be due to the uneven distribution of CaM in the cell. The actions of ryanodine and caffeine take place on the endoplasmic reticulum in the cell body (*15*), whereas most of the photoreceptor's CaM is localized to the rhabdomere (*16*).
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29. The increase of $[Ca^{2+}]_i$ in M5-treated cells was observed only before M5 abolished the LIC (usually <2 min after the formation of whole-cell recordings). This biphasic effect of M5 on the LIC (initial enhancement followed by disruption) can be explained by assuming that Ca^{2+} release from internal stores is required for light excitation. Accordingly, when the concentration of Ca-CaM is reduced by M5 and Ca^{2+}-free medium, the negative feedback of Ca-CaM is relieved, resulting in an enhanced and uncontrolled release of Ca^{2+} from the internal stores followed by total store depletion. The failures to detect release of Ca^{2+} induced by light (*12–14*) were most likely due to a signal-detection problem.

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