A *Drosophila* mutant defective in extracellular calcium-dependent photoreceptor deactivation and rapid desensitization

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CALCIUM is involved in the adaptation of vertebrate photoreceptors to light[1,2] and may have a similar role in invertebrate phototransduction[3,4]. But the molecular mechanisms mediating this stimulus-dependent regulation are not well understood in any G protein-coupled transduction system. We have developed a preparation of isolated *Drosophila* photoreceptors that has allowed us to carry out an electrophysiological characterization of the light-activated response in these sensory neurons using patch-clamp techniques. We report here that extracellular calcium entering through the light-activated conductance is a key regulator of both the activation and deactivation phases of the phototransduction cascade, and that *inaC* mutant photoreceptors[5] are specifically defective in the calcium-dependent deactivation mechanism. These data suggest that the light-dependent calcium influx inactivates this cascade through a biochemical pathway that requires the *inaC* gene product, and that this mechanism represents a molecular basis for stimulus-dependent regulation of visual transduction in *Drosophila* photoreceptors.

The compound eye of *Drosophila* is composed of 800 ommatidia, or unit eyes, each of which contains eight photoreceptor neurons[6]. These eight cells can be divided into three classes: R1–R6, R7 and R8 (ref. 7). R1–R6 cells express a blue-absorbing rhodopsin, Rh1 (refs 8, 9), and are the major photoreceptor cell class in the retina. We have focused on the analysis of R1–R6 cells, as they are the best characterized class of photoreceptors with respect to signal transduction, and are the most experimentally accessible cell type. *Drosophila* photoreceptors were dissociated from the heads of late pupae 1–5 h before eclosion (stages p14, p15)[10] in clusters of R1–R8 cells, each of which represents one ommatidium stripped of all support and pigment cells (Fig. 1). A similar preparation from *Drosophila* adults has been described by Hardie *et al.*[11]. The R1–R6 cell soma are easily identified by the unique location of their nuclei at the very distal margin of each cluster (Fig. 1*c*).

Short flashes of white light (10-ms duration) used to stimulate dark-adapted photoreceptors evoke large currents with an average latency of 24.9 ± 2.3 ms ($n = 16$) (Fig. 1*d*), which under strong stimulation can reach more than 20 nA at peak amplitude. Table 1 summarizes a series of ion-substitution experiments demonstrating that the *Drosophila* light-activated channel is a cation-selective channel that can pass even large monovalent ions such as TEA^+ and $Tris^+$, suggesting a fairly large pore size. In addition, raising the extracellular Ca^{2+} concentration ($[Ca^{2+}]_{out}$) to 20 from 0.1 mM resulted in a +28 mV change in the reversal potential, indicating that Ca^{2+} passes through the pore with a high relative permeability.

It is significant that Ca^{2+} permeates this light-activated conductance because Ca^{2+} can act as a messenger mediating adaptation in vertebrate phototransduction[12–14] and may also be important in both excitation[15,16] and regulation of invertebrate phototransduction[17–19]. Pulses of light (200 ms or 1 s duration; Fig. 1*e*, *f*) evoke complex responses that have biphasic kinetics resulting from a rapid partial desensitization (attenuation of current) during the stimulus. The responses both to flashes and to pulses of light show a marked asymmetry in the kinetics of the light-activated currents about the reversal potential. For example, the kinetics of the rapid desensitization (as defined above) and deactivation (recovery of the light-activated response after termination of the stimulus) are much faster during inward current flow (Fig. 1*d*, *e*). The asymmetries in the kinetics of the light-activated currents seem not to be directly voltage dependent, but instead to be dependent on the direction of ion flow. Thus shifting the reversal potential under standard extracellular calcium concentrations (1 mM) simply causes the same asymmetries to be reflected around the new reversal potential (data not shown). Similar results obtained with the use of the nystatin perforated-patch technique[20] (Fig. 1*f*) demonstrate that these asymmetries are not the result of nonphysiological alterations of the intracellular environment during whole-cell recording. These results suggest a model in which extracellular calcium entering through the light-activated conductance may regulate the signalling cascade and may be responsible for the rapid kinetics of deactivation and desensitization of the response.

As this model predicts that the kinetics of the light response should be a function of $[Ca^{2+}]_{out}$, we examined the effects of different concentrations of extracellular calcium on the rates of the activation (defined as rate of onset of current after stimulus) and deactivation processes. Varying $[Ca^{2+}]_{out}$ from 1 mM to 20 mM causes a large increase in the rate of both activation and deactivation of the inward currents, but has a much smaller effect on the outward currents (Fig. 2*b*, *c*). In addition, both the rate and the degree of the rapid desensitization during a sustained light stimulus are greatly increased when the photoreceptors are exposed to 20 mM $[Ca^{2+}]_{out}$ (compare Fig. 3*b* with *c*). These changes are expected to affect predominantly the inward currents if only inward currents carry large amounts of Ca^{2+} into the cell, and this calcium entry then regulates the activation, deactivation and desensitization processes. During the outward currents, the calcium influx into the cell is presumably greatly attenuated both by the smaller electrochemical driving force and the opposing bulk flow of monovalent ions. For quantitative evaluation of the inward current data, the rate of activation was measured as the time to 50% of peak current from onset of the light stimulus, and the rate of the deactivation was measured

TABLE 1 Summary of ion substitution experiments

Bath $[Ca^{2+}]$ (mM)	Bath monovalents	Pipette monovalents	E_{rev}(mV)
0.1	124 mM CsCl	124 mM CsCl	−0.82 ± 0.75
0.1	124 mM NaCl	124 mM CsCl	−3.33 ± 0.4
0.1	124 mM KCl	124 mM CsCl	−2.18 ± 1.03
0.1	124 mM Tris-Cl	124 mM CsCl	−9.79 ± 0.43
0.1	124 mM TEA-Cl	124 mM CsCl	−24.76 ± 2.77
0.1	90 mM Cs-gluconate, 34 mM CsCl	124 mM CsCl	−0.981 ± 0.606
20	124 mM CsCl	124 mM CsCl	+28.74 ± 1.43

Pipette solution contained 124 mM CsCl, 10 mM HEPES, 11 mM EGTA, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 3 mM Mg^{2+}–ATP, 0.5 mM Na^+–GTP, pH 7.15 with CsOH. Bath solutions contained 124 mM monovalents as indicated, 10 mM HEPES, $CaCl_2$ as indicated, 32 mM sucrose, pH 7.15. Each value of E_{rev} is the average ± standard deviation of data from at least three cells. Reversal potentials were calculated from plotting peak currents against holding potential. The sequence of ion permeabilities was determined from reversal potentials using the Goldman–Hodgkin–Katz[28,29] equation simplified for the case of two permeant ions at the same intracellular(*i*) and extracellular(*o*) concentration: $E_{rev} = RT/zF(\ln(P_o/P_i))$. Relative permeabilities of ions: $P_{Ca^{2+}} > P_{Cs^+} > P_{Na^+} \sim P_{K^+} > P_{Tris^+} > P_{TEA^+}$.

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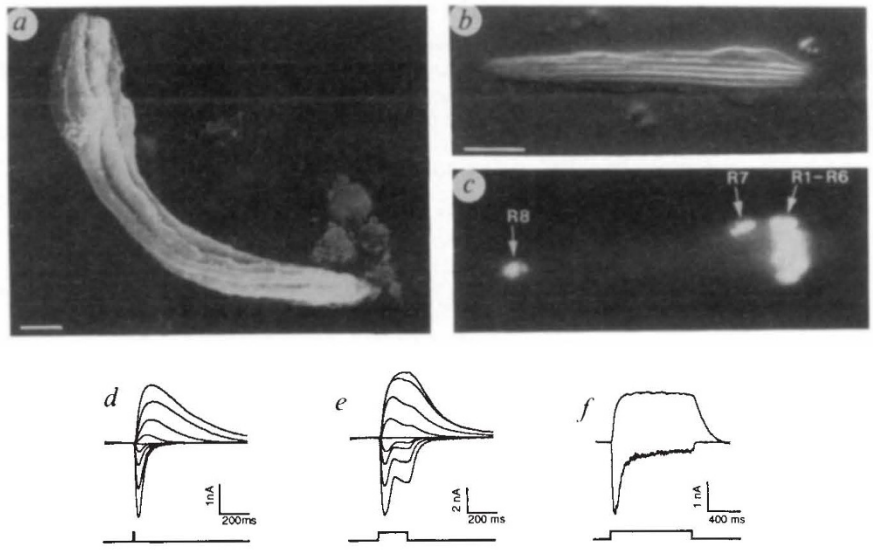

FIG. 1 Isolated *Drosophila* photoreceptor clusters, each representing one ommatidium, and whole-cell voltage-clamp recordings of light-activated currents. *a*, Scanning electron micrograph (SEM) showing that each cluster comprises a group of tightly packed individual photoreceptors. Scale bar, 10 μm. *b*, Normarski interference contrast image of a photoreceptor cluster; the distal end of the photoreceptors is to the right. Scale bar, 25 μm. *c*, The same cluster as in *b*, stained with a fluorescent marker specific for nuclei. The arrows indicate the position of the R1–R6, R7 and R8 nuclei. *d*, Light-activated currents evoked by 10 ms flashes of white light at holding potentials from −80 mV to +80 mV. Leak subtractions were done off-line, and all traces were normalized to zero at the pretrigger region just before onset of the light stimulus. Recordings of light-activated currents were made in symmetric Cs^+ solutions (to block voltage-activated K^+-channels, and aid in the isolation of the light-activated currents) with 1 mM Ca^{2+} in the bath. *e*, Light-activated currents evoked by 200-ms pulses of white light with the same protocol as in *d*. *f*, Light-activated currents evoked by 1-s pulses of white light at −80 mV and +80 mV holding potentials. Records in *f* were obtained with the use of the nystatin perforated patch technique. All light-activated responses were obtained from different cells (in all figures).

METHODS. Heads were dissected from stage p15 wild-type Canton S *Drosophila* pupae, and rapidly chopped in 5 μl Ca^{2+}-free, Mg^{2+}-free Ringer's solution (120 mM NaCl, 4 mM KCl, 10 mM HEPES, 32 mM sucrose, pH 7.15) on a glass plate. The resulting suspension was then transferred to about 30 μl of the same solution, and gently triturated through a pipette tip. All manipulations were done under dim red lights to prevent photoreceptor desensitization. SEM was done using standard techniques. For fluorescent labelling of nuclei, the mixture was fixed in 2% glutaraldehyde for 10 min, incubated with 0.1% saponin (Sigma) for 1 min and incubated with 100 ng ml^{-1} Hoechst 33258 nuclear stain. Isolated photoreceptor clusters were immediately photographed without further manipulation. All processing steps were done at room temperature (23–25 °C). For electrophysiology, dissociated photoreceptor clusters were immediately allowed to settle under the bath solution onto a clean glass coverslip forming the bottom of a 35-mm-diameter recording dish. The dish was mounted onto the stage of a Leitz Fluovert inverted microscope, and cells were visualized through Hoffmann interference contrast under weak red-light illumination. The isolated photoreceptor clusters are stable in culture for several hours. In the whole-cell recording configuration, most late pupal photoreceptors had input resistances of 200–1,500 MΩ, and whole-cell capacitances of 35–50 pF. Recordings (23–25 °C) were made with patch pipettes made from fibre-filled borosilicate glass (Sutter Instruments) with average resistances of 5 MΩ. Junction potentials were nulled just before seal formation, and no changes in the ion composition of the bath were made after seal formation. Whole-cell recordings were made using standard techniques[28]. Perforated-patch recording techniques were adapted from ref. 20. Recordings were made with a 50 mg ml^{-1} stock nystatin (Sigma) solution in DMSO freshly diluted 1:500 into filtered pipette solution for each pipette. Pipette tips were filled with nystatin-free solution to aid in seal formation. Most (80%) series-resistance errors were compensated during recording, and data was only acquired from cells with seal resistances >5 GΩ. Signals were amplified with an Axopatch 1-D patch-clamp amplifier (Axon Instruments), filtered through an 8-pole Bessel filter at 2 kHz, and digitized at 125 kHz for analysis. Data were analysed using pClamp 5.5.1 software (Axon Instruments). Photoreceptors were stimulated with light from a 75 W Xenon arc lamp, attenuated using neutral density filters (Oriel), regulated by an electronic shutter and focused through the microscope objective. Physiological electrode solution: 120 mM KCl, 4 mM NaCl, 10 mM HEPES, 11 mM EGTA, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 3 mM Mg^{2+}–ATP, 0.5 mM Na^+–GTP, pH 7.15 with KOH. Physiological bath solution: 120 mM NaCl, 4 mM KCl, 10 mM HEPES, 1 mM $CaCl_2$, 32 mM sucrose, pH. 7.15 with NaOH. Symmetric Cs^+ solutions were as above, except that pipette and bath monovalent cations were replaced with 124 mM CsCl.

as the time from peak response to 75% attenuation of the peak current. Figure 2*g* and *h* shows these measurements plotted against $[Ca^{2+}]_{out}$ (solid bars). The results indicate that it is possible to manipulate the kinetics of activation, deactivation and desensitization of the light-activated response as a function of $[Ca^{2+}]_{out}$, thus demonstrating that extracellular calcium is sufficient to regulate the light response.

To demonstrate the necessity of extracellular calcium in these processes, we recorded from cells under conditions in which $[Ca^{2+}]_{out}$ is buffered with EGTA to $<10^{-8}$ M. In concordance with our model, these cells not only have extremely slow activation and deactivation rates, but now the kinetics of the currents become symmetric and independent of direction of ion flow (Fig. 2*a*). Additionally, the kinetics of these currents no longer show any biphasic characteristics in response to a pulse of light (Fig. 3*a*), suggesting that the rapid desensitization also requires extracellular calcium. Extracellular Ca^{2+} must enter the photoreceptor to regulate the light response, because buffering intracellular calcium levels with a fast calcium chelator, BAPTA (1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid) (ref. 21), instead of the slower EGTA, specifically reduces the rates of activation and deactivation (compare Fig. 3*c* with *f*). But the degree of response desensitization is similar to either calcium buffer, consistent with the fact that EGTA and BAPTA have similar dissociation constants for Ca^{2+}. Taken together, these results show that calcium influx through the light-activated conductance is required for regulating the light response.

To investigate the molecular bases of these calcium-dependent mechanisms, we screened known phototransduction mutants for those that may be deficient in these processes. Many of these mutants were isolated through mutagenic screens for flies with defective electroretinograms (ERG)[5,22]. ERGs are extracellular recordings of the electrical activity of the whole eye in response to light[23]. Figure 2*d*, *e* and *f* show the light-activated responses of homozygous *inaC* photoreceptors[5,24] at various $[Ca^{2+}]_{out}$. These photoreceptors are unable to carry out normal rapid deactivation after a light stimulus. By contrast, excitation mechanisms seem to be unaffected in this mutant, as the dependence of the rate of activation on $[Ca^{2+}]_{out}$ in *inaC* photoreceptors is similar to wild type (Fig. 2*g*, compare solid and dotted bars). To exclude the possibility that the *inaC* phenotype is caused by the inability of calcium to enter the photoreceptor cells, we showed that, as in wild-type photoreceptors, the reversal potential of the light-activated conductance in the mutant shifts with increasing $[Ca^{2+}]_{out}$ (not shown). The outward currents are largely unaffected by the mutation, suggesting that the expression of the mutant phenotype is dependent on the direction of ion flow. Figure 2*h* (dotted bars) shows the quantitative analysis of the inward current deactivation kinetics in *inaC* photoreceptors, demonstrating that *inaC* mutants are

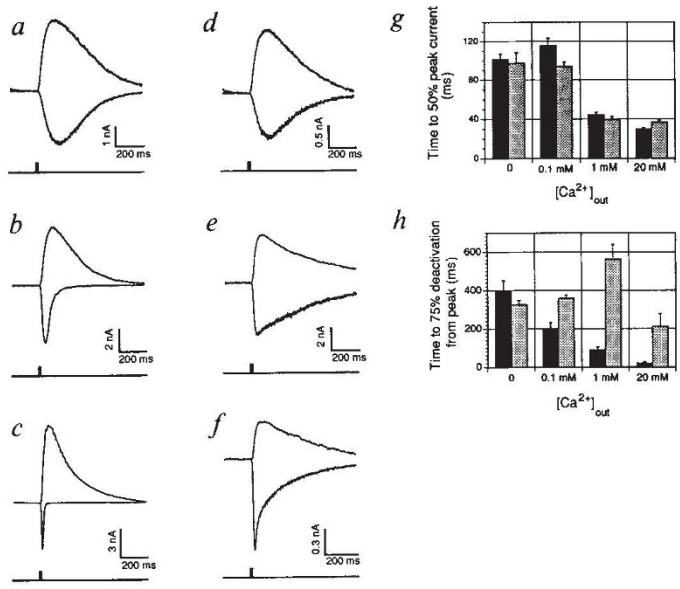

FIG. 2 Kinetics of activation and deactivation of the light-activated currents in wild-type and *inaC* mutant photoreceptors with varying $[Ca^{2+}]_{out}$. All photoreceptors were recorded under conditions of symmetric Cs^+ solutions (see Fig. 2 legend) with varying concentrations of extracellular calcium. *a, b, c,* Responses of wild-type cells with 0, 1 mM and 20 mM $[Ca^{2+}]_{out}$, respectively; *d, e, f,* responses of *inaC* mutant photoreceptors with 0, 1 mM and 20 mM $[Ca^{2+}]_{out}$, respectively. *a–f,* Recordings taken at holding potentials of −80 mV and +80 mV. *g,* Plot of time to 50% of peak current from onset of light stimulus for inward currents against $[Ca^{2+}]_{out}$. *h,* Plot of time from peak to 75% attenuation of the peak amplitude for inward currents against $[Ca^{2+}]_{out}$. Both graphs show data from wild-type (solid bars), and *inaC* mutant (dotted bars) photoreceptors. *g, h,* Recordings were done at a holding potential of −80 mV, and the stimulus consisted of a 10-ms flash of white light. Each data point consists of averaged data from at least five cells, and data from each cell consists of an average of at least nine individual traces.

METHODS. All wild-type data shown were acquired from Canton S photoreceptors. All mutant data shown were acquired from photoreceptors homozygous for either $inaC^{207}$ or $inaC^{209}$ alleles. All experiments were repeated with both alleles, and no phenotypic differences were observed between them (data not shown).

specifically defective in the calcium-dependent deactivation mechanism. Interestingly, *inaC* mutants are also defective in the rapid desensitization to longer light pulses (compare Fig. 3*b* and *e*). These findings suggest that Ca^{2+} influx as a result of channel activation causes rapid deactivation and desensitization through a regulatory pathway that depends on the *inaC* gene product.

To test for the requirement of extracellular calcium in regulating *inaC* activity, we compared the light response of mutant and wild-type photoreceptors under conditions in which

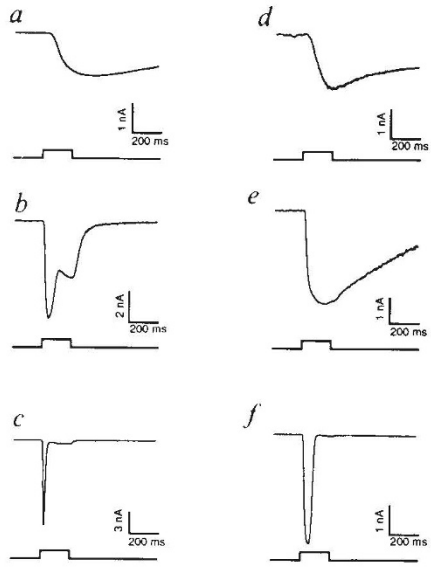

FIG. 3 Kinetics of desensitization during prolonged light stimuli in wild-type and *inaC* mutant photoreceptors with varying $[Ca^{2+}]_{out}$. *a, b, c,* Responses of wild-type photoreceptors recorded in symmetric Cs^+ solutions at a holding potential of −80 mV under conditions of 0, 1 mM and 20 mM $[Ca^{2+}]_{out}$, respectively. *d, e,* Responses of *inaC* photoreceptors recorded under the same conditions with 0 and 1 mM $[Ca^{2+}]_{out}$, respectively. The stimulus was a 200-ms pulse of white light. *f,* Response of a wild-type photoreceptor recorded under identical conditions as in *c* (20 mM $[Ca^{2+}]_{out}$, symmetric Cs^+ solutions) at a holding potential of −80 mV, except that buffering of Ca^{2+} levels in the pipette was with BAPTA (Molecular Probes) instead of EGTA. Pipette solution was as in Fig. 1 legend, except that EGTA was replaced with the same concentration of BAPTA.

$[Ca^{2+}]_{out}$ is buffered to $<10^{-8}$ M (Fig. 2*a, d, h*). Under these conditions, wild-type photoreceptors have deactivation kinetics similar to those of *inaC* cells. As the expression of the mutant phenotype requires the presence of extracellular Ca^{2+}, we conclude that wild-type *inaC* activity is dependent on the entry of extracellular calcium through the light-activated conductance.

Calcium-mediated mechanisms underlie many aspects of regulation of the visual cascade in vertebrate photoreceptors[1]. Recently, Ca^{2+}-binding proteins that seem to regulate guanylate cyclase[25,26] and cyclic GMP phosphodiesterase[27] have been purified and biochemically characterized. The ability to study *Drosophila* mutants with specific defects in calcium-dependent mechanisms involved in the regulation of the phototransduction cascade provides a powerful approach for the molecular investigation of these processes. □

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