Genetic Dissection of Light-induced Ca^{2+} Influx into *Drosophila* Photoreceptors

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ABSTRACT Invertebrate photoreceptors use the inositol-lipid signaling cascade for phototransduction. A useful approach to dissect this pathway and its regulation has been provided by the isolation of *Drosophila* visual mutants. We measured extracellular changes of Ca^{2+} $[\Delta Ca^{2+}]_o$ in *Drosophila* retina using Ca^{2+}-selective microelectrodes in both the transient receptor potential (*trp*) mutant, in which the calcium permeability of the light-sensitive channels is greatly diminished and in the inactivation-but-no-afterpotential C (*inaC*) mutant which lacks photoreceptor-specific protein kinase C (PKC). Illumination induced a decrease in extracellular $[Ca^{2+}]$ with kinetics and magnitude that changed with light intensity. Compared to wild-type, the light-induced decrease in $[Ca^{2+}]_o$ (the Ca^{2+} signal) was diminished in *trp* but significantly enhanced in *inaC*. The enhanced Ca^{2+} signal was diminished in the double mutant *inaC;trp* indicating that the effect of the *trp* mutation overrides the enhancement observed in the absence of eye-PKC. We suggest that the decrease in $[Ca^{2+}]_o$ reflects light-induced Ca^{2+} influx into the photoreceptors and that the *trp* mutation blocks a large fraction of this Ca^{2+} influx, while the absence of eye specific PKC leads to enhancement of light-induced Ca^{2+} influx. This suggestion was supported by Ca^{2+} measurements in isolated ommatidia loaded with the fluorescent Ca^{2+} indicator, Ca Green-5N, which indicated an approximately threefold larger light-induced increase in cellular Ca^{2+} in *inaC* relative to WT. Our observations are consistent with the hypothesis that TRP is a light activated Ca^{2+} channel and that the increased Ca^{2+} influx observed in the absence of PKC is mediated mainly via the TRP channel.

INTRODUCTION

Invertebrate photoreceptors use the phosphoinositide (PI) signaling cascade for phototransduction providing a useful model system to study this important and ubiquitous signaling cascade. (Fein, Payne, Corson, Berridge, and Irvine, 1984; Brown, Rubin, Ghalayini, Tarver, Irvine, Berridge, and Anderson, 1984; Devary, Heichal, Blumenfeld, Cassel, Suss, Barash, Rubinstein, Minke, and Selinger, 1987;

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Payne, Walz, Levy, and Fein, 1988; Bloomquist, Shortridge, Schneuwly, Pedrew, Montell, Steller, Rubin, and Pak, 1988; Minke and Selinger, 1991; Hardie and Minke, 1993). Detailed studies in *Limulus* ventral eye and bee photoreceptors have shown that a major function of inositol 1,4,5 tris phosphate ($InsP_3$) is to release Ca^{2+} from intracellular stores (Payne et al., 1988; Baumann and Walz, 1989). Changes in intracellular Ca^{2+} have been measured using variety of methods in several invertebrate species (Brown and Blinks, 1974; Brown, Brown, and Pinto, 1977; Payne, Corson, Fein, and Berridge, 1986; Levy and Fein, 1985; Brown, Rydquist, and Moser, 1988; Coles and Orkand, 1985).

The extensive research on PI-mediated Ca^{2+} mobilization (reviewed in Irvine, 1991; Berridge, 1993) can benefit greatly from the exploitation of *Drosophila* visual mutants for studying Ca^{2+} mobilization. Two *Drosophila* mutations have been recently shown to affect light-induced Ca^{2+} mobilization: the transient receptor potential (*trp*) mutation (Cosens and Manning, 1969, Minke, Wu, and Pak, 1975; Minke, 1982; Hardie and Minke, 1992, 1993) and the inactivation-but-no-afterpotential C (*inaC*) mutation (Pak, 1979; Smith, Ranganathan, Hardy, Marx, Tsuchida, and Zuker, 1991; Hardie, Peretz, Suss-Toby, Rom-Glas, Bishop, Selinger, and Minke, 1993). In the *trp* mutant, the receptor potential declines to baseline during prolonged illumination recovering ~ 1 min in the dark (Cosens and Manning, 1969; Minke et al., 1975). The *trp* gene product (Montell and Rubin, 1989; Wong, Schaefer, Roop, La Mendola, Johnson-Seaton, and Shao, 1989), which has the general structure of a Ca^{2+} channel subunit with some homology to the dihydropyridine receptor (Phillips, Bull, and Kelly, 1992) is required for the high Ca^{2+} permeability of the light-sensitive channels and presumably constitutes a PI-mediated Ca^{2+} influx pathway (Hardie and Minke, 1992). The *inaC* protein is a photoreceptor specific protein kinase C (PKC) (Smith et al., 1991). In contrast to the relatively simple phenotype of *trp,* the *inaC* mutant has a very complex phenotype including the following characteristics: (*a*) the initial fast decline of the peak receptor potential of WT to a lower steady state phase (within 30 ms) in response to *intense* light is almost absent in *inaC.* Instead, the receptor potential of *inaC* decays relatively slowly (within 1 min) towards an abnormally low steady state level whose amplitude decreases with increasing light intensity. (*b*) At light off, the termination of the receptor potential of *inaC* is abnormally slow. (*c*) Light adaptation, as measured by the shift of the intensity-response function and the decrease in response latency with the increase in background light, is largely reduced in *inaC.* (*d*) In whole-cell recordings, individual single photon responses (quantum bumps; Yeandle, 1958) terminate abnormally slowly. As a consequence, in response to continuous dim light, the slowly terminated phase of the bumps sum and produce a steady state response which is approximately four times larger relative to WT at normal $[Ca^{2+}]_o$ (e.g., 1.5 mM). This facilitation of the dim light steady state response in *inaC* is reversed if recordings are made after several minutes exposure to Ca^{2+} free Ringer combined with adapting light. Sensitivity to light is now greatly reduced, but if extracellular Ca^{2+} is transiently raised, the response is rapidly restored (for details see Hardie et al., 1993; Smith et al., 1991).

On the assumption that the quantum bump represents quantal release of Ca^{2+} from intracellular stores by $InsP_3$ and that Ca^{2+} is required for excitation, it was possible to account for the pleiotropic manifestations of the *inaC* phenotype as

arising from one primary defect: the failure of quantum bumps to terminate normally. Accordingly, when the Ca^{2+} stores are full, a failure to terminate the release of Ca^{2+} leads to increased release per photon and hence supersensitivity and abnormally long response termination. On the other hand, intense prolonged light causes depletion of Ca^{2+} from the stores leading to collapse of the response. This collapse is accelerated at zero external Ca^{2+} which facilitates depletion of the stores while sensitivity can be restored by readdition of Ca^{2+} to the medium which accelerates refilling of the Ca^{2+} stores (Hardie et al., 1993). In the present study, we tested some predictions of the above model (see Discussion).

One means of studying both cellular Ca^{2+} and the electrical response simultaneously in *Drosophila* has been provided by a new preparation which allows whole cell voltage clamp recordings to be made from photoreceptors in dissociated ommatidia (Hardie, 1991; Ranganathan, Harris, Stevens, and Zuker, 1991). This method has been recently extended to include internal dialysis with Ca^{2+} indicators (Peretz, Suss-Toby, Rom-Glas, Arnon, Payne, and Minke, 1994), allowing measurements of light-induced increase in cellular Ca^{2+}. However, this procedure requires very strong lights which usually result in irreversible damage in the whole-cell recording situation. A complementary, independent method for measuring Ca^{2+} mobilization in the intact eye is the use of Ca^{2+}-selective microelectrodes for measuring changes in external Ca^{2+}. It has been shown in several studies that, due to the small volume of retinal extracellular space in insects, large changes in Ca^{2+} concentration ($[\Delta Ca^{2+}]_o$) can be measured during and after light (Orkand, Dietzel, and Coles, 1984; Minke and Tsacopoulos, 1986; Ziegler and Walz, 1989; Sandler and Kirschfeld, 1988, 1991, 1992; Rom-Glas, Sandler, Kirschfeld, and Minke, 1992). The initial decrease in $[Ca^{2+}]_o$ was interpreted as light-induced Ca^{2+} influx into the photoreceptors and the subsequent return of $[Ca^{2+}]_o$ towards baseline during or after light was interpreted as due to activation of Na^+-Ca^{2+} exchanger, extruding Ca^{2+} from the cell (Minke and Tsacopoulos, 1986; Ziegler and Walz, 1989).

In the present study, we applied the Ca^{2+}-selective microelectrode technique for the first time to *Drosophila* retina. The decrease in $[Ca^{2+}]_o$ in response to light was significantly larger than similar signals previously reported in larger flies (Sandler and Kirschfeld, 1988, 1991; Rom-Glas et al., 1992). The application of this technique to the *Drosophila* mutants flies *trp* and *inaC* made it possible to examine the function of the *trp* and *inaC* proteins in the intact retina under physiological conditions. We show that the *trp* mutation diminishes the light-induced decrease in $[Ca^{2+}]_o$ in the retina. We also show that the *inaC* mutation enhances the light-induced decrease in $[Ca^{2+}]_o$, suggesting enhancement of Ca^{2+} influx into *inaC* photoreceptors relative to WT. This suggestion was supported by measurements of light-induced increase in cellular Ca^{2+} in WT and *inaC* using internal dialysis of a fluorescent Ca^{2+} indicator in whole-cell recordings of mutants and WT ommatidia.

MATERIALS AND METHODS

Fly Stocks

The wild-type (WT) strain was white-eyed (*w*) *Drosophila melanogaster* of the Oregon-R-strain. In addition, experiments were performed on the white-eyed transient receptor potential (trp^{CM})

and the inactivation-but-no-afterpotential C (*inaC*^{P209}, Pak, 1979; Smith et al., 1991) mutants. Flies were raised at 25°C.

Whole-Cell Patch Clamp Recordings

This method has been described in detail before (Hardie, 1991). Briefly, adult (<4-h old) or late p15 pupae of *Drosophila* ommatidia (Bainbridge and Bownes, 1981) were prepared as described previously (Hardie, 1991). Retinae were rapidly dissected in normal Ringer's solution, transferred to Ringer's solution supplemented with 10% fetal calf serum (FCS Ringer), and gently triturated with silanized glass pipettes fire polished to a diameter of ~100–150 μm. During the dissociation procedure, which requires no enzyme treatment, the surrounding pigment cells disintegrate, exposing the photoreceptor membrane. The procedure also results in the ommatidia breaking off at the basement membrane; thus, the photoreceptors lack their axon terminals. Ommatidia were used immediately after dissection. Aliquots (~10 μl) of ommatidia were allowed to settle in a small chamber, the bottom of which is formed by a clean coverslip, on the stage of an inverted microscope stage. Recordings were made at 21(± 1)°C, with patch pipettes pulled from borosilicate glass (fiber filled) with resistance of ~10 MΩ for whole-cell recordings. Whole-cell recordings were achieved using standard techniques (Hardie, 1991). Signals were amplified using an Axopatch-1D (Axon Instruments, Inc., Foster City, CA) patch clamp amplifier, filtered via a 4-pole Bessel filter, below 1 kHz.

Solutions

Bath Ringer contained (in millimolar): 120 NaCl, 5 KCl, 8 $MgSO_4$, 10 N-Tris-(hydroxymethyl)-methyl-2-amino-ethanesulphonic acid (TES), 25 sucrose, 5 proline, 1.5 $CaCl_2$. Pipette solutions: to block K channel activity, the pipette solution contained 120 CsCl 15 tetra-ethyl ammonium (TEA) Cl, 2 $MgSO_4$, 10 TES (junction potential − 3 mV) and Ca Green 5-N (Molecular Probes Inc., Eugene, OR) 100 μM.

Optical Setup and Photon Counting

A Zeiss Axiovert 35 inverted microscope (Carl Zeiss, Oberkochen, Germany) with an achroplan 40× objective (N.A. 0.6) was used for optical recordings. A high-intensity Xenon source was used to provide conventional epiillumination for measuring Ca^{2+}-indicator fluorescence and provided strong stimulation of the photoreceptors, while a tungsten transillumination source provided illumination for viewing the preparation and for weak stimulation of the photoreceptors.

Epiillumination for excitation of fluorescent dyes came from a 150 W Xenon light source (Zeiss XBO) filtered via a 395–485 nm band pass excitation filter and a 510-nm dichroic mirror, which reflected the light into the objective. An electromechanical shutter (model VS25S2TO, Vincent Associates, Rochester, NY) was placed in the path of the excitation light. A photodiode was used to monitor the opening time of the shutter (~6 ms). The fluorescent light from the specimen was filtered via a 510–565 barrier filter (Zeiss) and focused onto a photomultiplier attached to photon counting unit (Hamamatsu Photonics 649/C1050, Hamamatsu City, Japan). The photomultiplier was mounted on the trinocular head of the microscope and a prism was introduced, when needed, that diverted light from the eyepieces to the photomultiplier. The perfusion chamber used for this study has been described previously (Minke and Payne, 1991). Transillumination for viewing the preparation with dim red light was provided by a 100 W 12 V halogen lamp, in conjunction with a Schott 630 RG edge filter and 2 KG3 heat filters (Minke and Payne, 1991). The illumination was passed through a shutter and reflected by a prism into the microscope condenser.

Fluorescence Ca^{2+} Measurements

Giga seal formation was obtained under dim, red transillumination (see above). Rapid washing of the chamber after seal formation reduced the background fluorescence, due to indicator leakage from the pipette, to a minimum level. A stop was then placed in the focal plane of the objective in the light path leading to the trinocular head of the microscope, limiting the field of view of both the observer and the photomultiplier to the ommatidium and the tip of the recording/perfusing pipette. A loading time of 1 min in the dark at zero holding potential was allowed to fill the cell with dye, followed by a clamp of the membrane potential to −50 mV resulting in a small capacitive current observed in the traces. 100 ms after the end of the loading period, the epiillumination system with the Xenon source (see above) was used to deliver a 5-s flash of relative intensity log 0.3. The resulting LIC and fluorescence signal were recorded simultaneously. After this intense illumination, the cell's sensitivity to light did not recover before spontaneous activation of an inward current occurred (run down; see Hardie and Minke, 1994). Thus, only one response could be measured from a given ommatidium, although additional responses were measured from other ommatidia of the same retina. The intense excitation light was essential for recording a reliable Ca^{2+} signal. To keep the cells of the isolated ommatidia responsive, a >1,000-fold attenuation in excitation light intensity was required. However, the Ca^{2+} signal was reduced to noise levels under such conditions. After measurement of fluorescence, the ommatidium was removed from the field of view and an identical stimulus was applied in the absence of the ommatidium in the field of view. The resulting background fluorescence was much smaller than that observed in the presence of the ommatidium. The same procedure was repeated for several other ommatidia of the same retina with reproducible results. Experiments showing no, or smaller than usual LIC (due to the use of young pupae) or a large background fluorescence (due to some leak of dye from the pipette or the cell) were rejected.

Intracellular Recordings from Intact Drosophila

After immobilizing the fly with low melting point wax, a razor blade chip was used to cut a small shallow triangular hole (sides 5–10 ommatidia long) in the cornea of the dorsal part of the compound eye without cutting any ommatidia (see below) and the surface was covered with petroleum jelly. The piece of cornea was carefully removed. The fly was grounded via an indifferent glass electrode containing fly Ringer's solution which was placed on the cornea of the same eye in a drop of electrode gel. Intracellular receptor potentials were measured with standard glass micropipettes filled with 2 M KCl (electrical resistance 80–140 MΩ), introduced through the corneal opening.

Ca^{2+} Measurements Using Ca^{2+}-selective Microelectrodes

The basic method applied to the large flies has been described in detail previously (Sandler and Kirschfeld, 1988, 1991; Rom-Glas et al., 1992). The *Drosophila* preparation was identical to that for intracellular recordings. Briefly, Ca^{2+}-selective microelectrodes were pulled from borosilicate double-barreled capillaries with filament (KBF 215075, Zak, D-8000 Munchen, Germany), and the tip broken off to an outer diameter of 2.0–5.0 μm. The reference barrel was filled with 0.2 M NaCl solution. For silanization of the ion-selective barrel, a column of 5% trimethylchlorsilane solution in CCl_4, several hundred micrometers long, was sucked into its tip and pushed out again several times (silanization time 3–5 min). The ionophore (neutral carrier ETH 1001, calcium ionophore I—cocktail A: Art.-Nr. 21048, Fluka Chemical Co., Neu-Ulm, Germany) was then introduced into the pipette (70–700 μm), after which a layer of saline solution (0.1 M $CaCl_2$) was applied from the unprocessed end of the electrode. A bridge to the differential amplifier (J. Meyer, Munchen, Germany), was formed by chloridized silver wires glued into the

electrode with dental wax. These ion-selective microelectrodes were produced by a slight modification of the methods given by Lux and Neher (1973) and Oehme, Kessler, and Simon (1976). The electrical resistance of the active channel was between 3 and 25 GΩ. When immersed alternately in two calibration solutions, containing 200 mM NaCl plus either 1.6 or 1.1 mM $CaCl_2$, the electrical potential recorded by the Ca^{2+}-selective microelectrode reached 90% of its final value in <0.8 s. These electrodes were found to be highly selective for Ca^{2+}. For 12 Ca^{2+}-selective microelectrodes, the selectivity coefficients with respect to K^+ and Na^+ were determined by the "fixed interference" method (Guilbault, 1976; see also Sandler and Kirschfeld, 1991). The Ca^{2+} buffer solutions required for this calibration were basically the same as used by Tsien and Rink (1980); as fixed ionic components they contained either 125 mM K^+ or 90 mM Na^+ plus 35 mM K^+, while the variable component had a free Ca^{2+} concentration of 10^{-2} to 10^{-8} M. The values of the potentiometric selectivity coefficients ($K_{CaK} = 10^{-5.10}$, $K_{CaNa} = 10^{-4.35}$) can be calculated from the concentration at the limit of detection (Guilbault, 1976; Ammann, 1986).

The Dark Value of the Retinal $[Ca^{2+}]_o$ in WT and the inaC Mutant

The dark values of retinal $[Ca^{2+}]_o$ were determined with Ca^{2+}-selective microelectrodes by placing a droplet of standard Ringer's solution used for electroretinogram recordings (1.4 mM $CaCl_2$) over the electrode insertion site and measuring the difference in the electrode potentials as the tip is moved back and forth between retina and droplet. Knowing the absolute $[Ca^{2+}]_o$ in the dark, gives the Ca^{2+} concentration changes upon illumination. $[Ca^{2+}]_o$ was calculated by means of the Nernst equation using the dark values and the light-induced potentiometric potential changes [ΔE_{Ca}] of the calcium electrode. In 3 WT *Drosophila* $[Ca^{2+}]_o$ was determined to be 1.43 mM ± 0.04 mM (SEM, eight measurements in each fly) and in 3 *inaC* mutants the $[Ca^{2+}]_o$ was 1.31 ± 0.01 mM. These results indicate that the $[Ca^{2+}]_o$ in the dark in *Drosophila* is very similar to that found in the larger flies (Sandler and Kirschfeld, 1988; Rom-Glas et al., 1992).

Light Stimuli

White light from a Xenon high-pressure lamp (XBO, 75 W) was delivered to the compound eye in a shielded cage by means of a fiber optic and diffuser. The maximal luminous intensity at the eye surface was ~1 cd/cm², which is ~2.5 logarithmic intensity units above the intensity for a half-maximal response of the most common type of fly photoreceptors (type R1-6). This effective light intensity is similar to the effective light intensity of the excitation light in the fluorescent microscope used for Ca^{2+} and light-induced current (LIC) measurements. The light intensity could be reduced by up to eight orders of magnitude by gray filters (MTO Massy, France) mounted on two electronically controlled filter wheels.

Experimental Setup

The outputs of the differential amplifier were connected to a storage oscilloscope (Tektronix 5111), a chart recorder (Hewlett Packard 7402A) and a laboratory computer (IBM-compatible personal computer AT10-4). For measurements with ion-selective microelectrodes, low-pass filters were inserted before the chart recorder and the computer (corner frequencies 100 Hz for the extracellular potentials and 10 Hz for the ion trace). A measurement card (DT 2801-A) and special-purpose software programmed in Turbo Pascal G. O. (Borland International, Munchen, Germany), enabled the recorded signals to be fed to the computer for further analysis. The extracellular calcium concentration ($[Ca^{2+}]_o$) was measured by first applying a tiny drop of Ringer to the corneal insertion site, by means of a syringe mounted on a micromanipulator. The solution contained 140 mM NaCl, 4 mM KCl, 10 mM Tris (pH 7.4) and 1.4 mM $CaCl_2$.

When the Ca^{2+}-selective microelectrode was withdrawn from the retina into the droplet of Ringer, a potential difference was recorded, from which the retinal $[Ca^{2+}]_o$ could be calculated. To measure light-induced extracellular Ca^{2+} concentrations changes, the eye was illuminated for 1 min. In these experiments, the interstimulus interval was at least 2 min. Because of the high selectivity of the Ca^{2+} electrodes, there is no relevant error in $\Delta[Ca^{2+}]_o$ if one neglects physiologically induced K^+ and Na^+ concentration changes in the fly retina during light stimulation. Furthermore, the resulting errors due to changes of the liquid-junction potential between the reference electrolyte of the Ca^{2+} electrode and the retinal fluid are also small. Neglecting these factors overestimated $\Delta[Ca^{2+}]_o$ by up to 3.5% for the light-induced plateau values and between 9 and 18% for the peak values, the so-called light minimum, from the maximal change measured (Sandler and Kirschfeld, 1991).

Monitoring of Damaged Tissue

To measure any damage caused to the retinal tissue by the Ca^{2+} electrode we applied the Lucifer yellow CH staining method which detects damaged cells (Wunderer, Picaud, and Francechini, 1989). A drop of Lucifer yellow CH (L-453, Molecular Probes, Inc., 1% in Ringer's solution) was applied to the eye after insertion of the Ca^{2+} electrode into the cut. The fly was kept in the dark for 30 min to allow binding of the dye to intracellular proteins in damaged cells. Then, the entire eye was sliced from the head under dim red light and washed in the dark in Ringer's solution for 60 min. The eye was fixed in formaldehyde 4% overnight at 4°C and then washed in 0.1 M cacodylate buffer with 5% sucrose. Eyes were then rinsed three times (10 min each) in 0.1 M cacodylate buffer (pH 7.2). The eyes were dehydrated in an ethanol series (in the range of 30–100%), embedded in Spurr's epoxy resin and sectioned for light microscopy (3-μm thick sections). As a positive control for ommatidial damage, we made a deeper (50 μm) cut (using vibrating razor blade) in the retinal tissue making a smooth horizontal cut without removing any tissue. We repeated the staining procedure (without inserting any electrodes) as described above. The Lucifer yellow CH has been shown to stain only damaged cells or photoreceptors illuminated with intense lights (Wunderer et al., 1989). Observation of serial cross-sections (3 μm thick) at various levels of the eye to which the electrode was inserted revealed no fluorescence staining of the ommatidia. Only the periphery of the sliced cornea and adjacent nonphotoreceptor cells were stained. In contrast, all sectioned ommatidia in the positive control experiments were heavily stained. This result indicates that the Ca^{2+} electrode caused minimal damage to the retina. The 3-μm recording electrode presumably pushed aside the flexible ommatidia without tearing any cells. Furthermore, even if some dead space was formed by accumulation of hemolymph around the electrode this is not expected to affect our measurements even if the dead space is as large as 10 μm in diameter (Lux and Neher, 1973; see Discussion).

RESULTS

The trp Mutation Diminished the Light-induced Decrease of $[Ca^{2+}]_o$

In the retina of intact WT *Drosophila,* prolonged intense light elicited a transient decrease in $[Ca^{2+}]_o$ reaching a peak in ~3 s followed by a partial return of $[Ca^{2+}]_o$ towards baseline (Fig. 1, *left*). This light-induced Ca^{2+} signal is similar to the Ca^{2+} signal measured previously in the larger flies *Calliphora* (Sandler and Kirschfeld, 1988, 1991, 1992) and *Lucilia* (Rom-Glas et al., 1992) except that it was significantly larger in *Drosophila.* The overshoot in the Ca^{2+} signal at light off was small compared to measurements in the honeybee drone (Minke and Tsacopoulos, 1986) or the

larger flies (Ziegler and Walz, 1989; Sandler and Kirschfeld, 1988). The extracellularly recorded receptor potentials (*fp, WT;* Fig. 1, *left*) were typical for the fly and very similar to records obtained in the larger fly *Calliphora* (Sandler and Kirschfeld, 1988, 1991). Large number of intracellular recordings in intact *Drosophila* eye under stimulation and recording conditions, very similar to those of the present study indicated that the general wave form of the field potential was similar to the

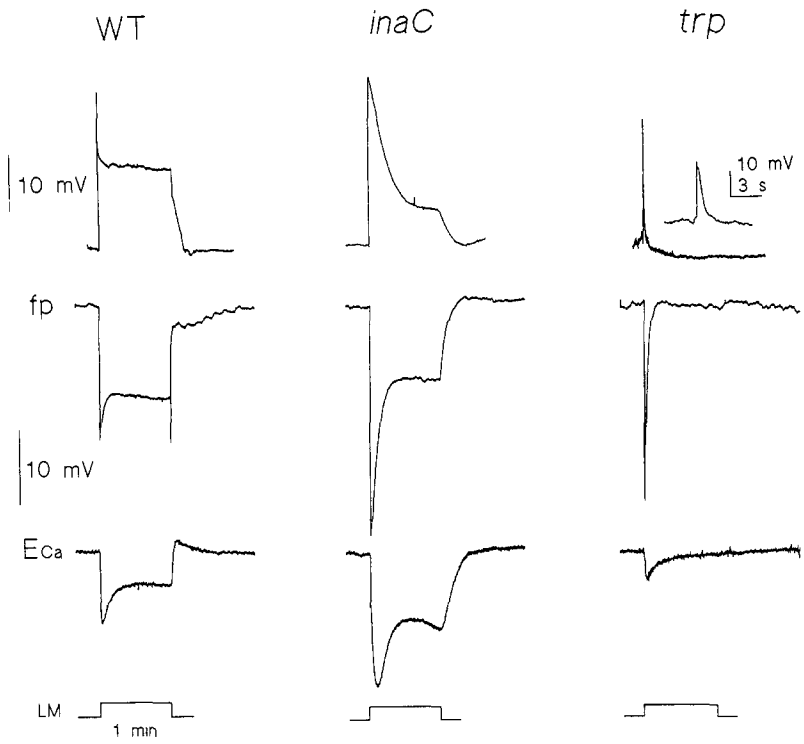

FIGURE 1. Light-induced $[Ca^{2+}]$ changes in the retinal extracellular space of white-eyed normal (WT) and white-eyed mutants *inaC* and *trp* of *Drosophila*. (*Left*) Recordings from an intact WT eye and the middle and right columns from $inaC^{P209}$ and trp^{CM} eyes under identical conditions. The top and middle rows are the intracellularly and extracellularly (field potential: *fp*) recorded receptor potentials. The third row is the light-induced potentiometric potential changes (ΔE_{Ca}) of the calcium-selective barrel of the Ca^{2+} microelectrode. The bottom traces give the time scale and the light monitor (*LM*) (in Figs. 1–3). The relative light intensity was log-1 for all traces. (*Inset*) Upper right corner shows the receptor potential of *trp* at faster time scale. The figure shows that ΔE_{Ca} is reduced in *trp* and enhanced in *inaC*.

intracellularly recorded receptor potential except that the ratio of the peak transient to the following steady state phase was somewhat different in the two electrical recordings configurations (see Fig. 1).

In contrast to the Ca^{2+} signal of WT flies, the decrease in $[Ca^{2+}]_o$ observed in *trp* retina under virtually identical stimulation and recording conditions was relatively small and only transient (Fig. 1, *right*). The intracellular and extracellular receptor

potential in *trp* showed the typical transient response to prolonged intense lights. The intracellular recordings of the receptor potential in *trp* showed a fast decline of the receptor potential to below zero baseline within 2 s (Fig. 1, *inset*). This result was similar to that obtained in the no steady state (*nss*) mutant of *Lucilia* (Rom-Glas et al., 1992) which is most likely the *trp* homolog mutation found in the larger flies (Howard, 1984; Barash, Suss, Stavenga, Rubinstein, and Minke, 1988).

The Ca^{2+} Signal Was Enhanced by the inaC Mutation

Fig. 1 also compares the intracellularly recorded receptor potential of WT (*left*) and *inaC* (*middle, top trace*). In response to intense light the intracellularly recorded response of WT was typically composed of an initial fast transient (8 ms time to peak) which declined to a lower steady state phase (within 33 ms), a manifestation of the process of light adaptation (Lisman and Brown, 1972). In *inaC* in contrast, the initial rapid transient was very small or absent and the response decayed slowly towards a relatively small steady state level within ~30 s. At light off, a hyperpolarizing phase was sometimes observed. The magnitude of this phase was variable in different cells, probably because of some damage caused to the cell by the penetrating microelectrodes. This phase may reflect the activation of the electrogenic Na^+-K^+ pump (Koike, Brown, and Hagiwara, 1971; Lisman and Brown, 1972). A similar behavior was observed by extracellular recording of the field potential (*fp, second row*). Quantitative differences between the fp and the intracellularly recorded receptor potential were not the topic of this study. Therefore, we did not investigate these differences further but used the fp as a monitor of the viability of the retina. The Ca^{2+} signal (*third row*) had in general, a similar wave form in both WT and *inaC*. The significant differences were: (*a*) The Ca^{2+} signal obtained in *inaC* was larger than that in WT. (*b*) The Ca^{2+} signal of *inaC* was slower relative to WT. (*c*) At medium light intensities (log-3, log-2, see Fig. 2) a significantly larger overshoot was observed at light off in *inaC* relative to WT while this overshoot was abolished at higher light intensities (Fig. 2).

To compare the dependence of the Ca^{2+} signal on light intensity in WT and *inaC*, $[Ca^{2+}]_o$ was measured under identical conditions in intact WT and *inaC* flies. Fig. 2 shows an example of measurements from a single retina of WT and *inaC*. The Ca^{2+} signal in *inaC* was much larger than that recorded in WT at all light intensities. The difference was particularly pronounced at log-3 light intensity where the peak amplitude of E_{Ca} was ~2.5 times larger in *inaC* relative to WT (see Fig. 4 for a quantitative comparison based on measurements in 39 flies).

The Ca^{2+} Signal of inaC Was Diminished in inaC;trp Double Mutant

To examine the epistatic relationship between the *inaC* and *trp* mutations, we measured light-induced changes in $[Ca^{2+}]_o$ in the double mutant *inaC;trp* (Hardie et al., 1993; Fig. 3). Fig. 3 shows that the *trp* mutation diminished the enhanced Ca^{2+} signal in *inaC*. Qualitatively, the *inaC;trp* phenotype was very similar to that of *trp;* however, closer examination revealed significant quantitative differences. The Ca^{2+} signal and field potential already appeared at dim lights in *inaC;trp* when no response was found in *trp*. At medium light intensities, the Ca^{2+} signal in the double mutant

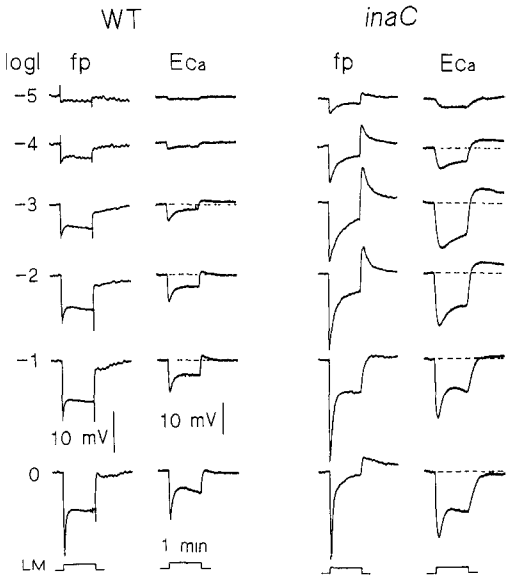

FIGURE 2. A comparison of the light-induced *fp* and ΔE_{Ca} recorded by the same double barrel electrodes in both *WT* and *inaC*. The figure shows that both the extracellularly recorded receptor potential and the light-induced reduction in extracellular Ca^{2+} (ΔE_{Ca}) are significantly larger and slower in *inaC* relative to WT at all light intensities. A pronounced overshoot of the Ca^{2+} signal after light off with medium but not with intense lights, is observed in *inaC* and not in *WT*. The horizontal dotted line indicates the resting $[Ca]_o$ in some traces.

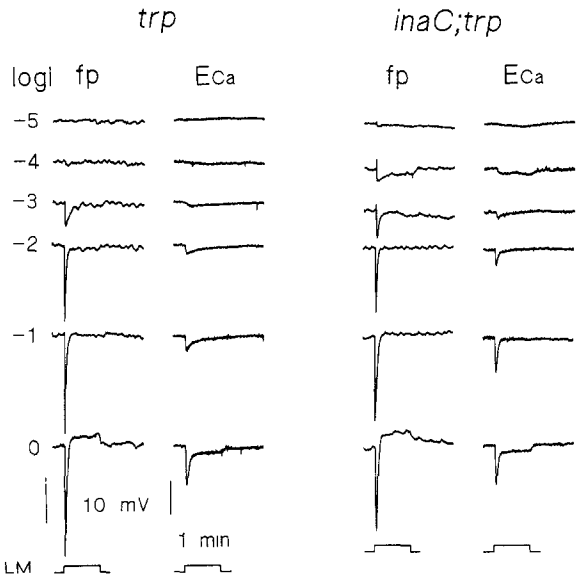

FIGURE 3. Light-induced fp and ΔE_{Ca} in the *trp* mutant and the double mutant *inaC;trp*. The experimental procedure and presentation are as in Fig. 2. Field potentials with similar wave forms are recorded in both *trp* and *inaC;trp* however, the Ca^{2+} signal is significantly larger in the double mutant *inaC;trp* relative to *trp* with intense lights (see Fig. 4 *A*).

The figure shows a comparison of the light-induced field potential and ΔE_{Ca} in a single retina of *trp* (*left*) and *inaC;trp* double mutant (*right*) at various light intensities as indicated. The field potential and Ca^{2+} signal are already observed the *inaC;trp* at light intensities which are at least 10 times dimmer than the light intensities required to elicit detectable responses in *trp* eyes. Also the Ca^{2+} signal is larger at intense lights in the double mutant relative to the *trp* mutant. The figure shows that the *trp* mutation largely reduced the Ca^{2+} signal observed in both WT and *inaC*.

was larger than in *trp* and decayed faster (Fig. 3). The smaller $\Delta[Ca^{2+}]_o$ observed in *trp* relative to WT was also found in the double mutant *inaC;trp* at all light intensities.

Light-induced Decrease in $[Ca^{2+}]_o$ Was Significantly Enhanced in inaC and Diminished in trp

Fig. 4, based on measurements in 39 flies, summarizes the light-induced changes in $[Ca^{2+}]_o$ as a function of relative light intensity both at the time of the peak decrease in

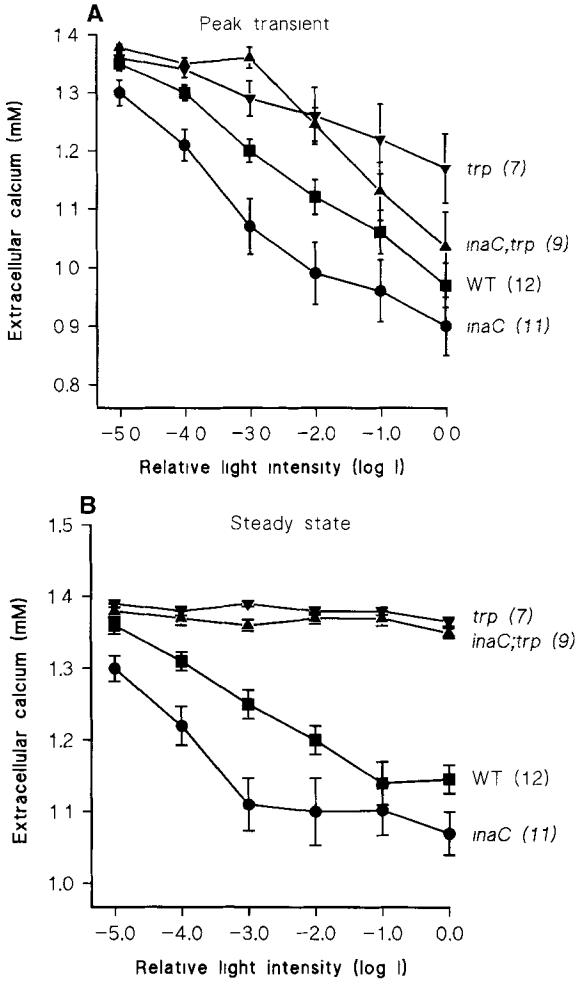

FIGURE 4. (*A*) The dependence of the peak decrease in extracellular $[Ca^{2+}]$ on light intensity in WT (n = 12), *inaC* (n = 11), *trp* (n = 7), and *inaC; trp* (n = 9) flies. Extracellular $[Ca^{2+}]$ was calculated using the Nernst equation. The $[Ca^{2+}]_o$ in the dark was measured directly for each mutant (see Materials and Methods). The error bars are standard deviation of the mean. The figure shows that the peak $\Delta[Ca^{2+}]_o$ is the largest in *inaC* retina at all light intensities. It furthermore shows that the *trp* mutation greatly diminishes the peak $\Delta[Ca^{2+}]_o$ of both WT and *inaC* flies. (*B*) The dependence of the decrease in steady state amplitude of $[Ca^{2+}]_o$, in the experiments of Fig. 4 *A*, on light intensity. The steady state decrease in $[Ca^{2+}]_o$ (measured 60 s after light onset) was almost abolished in the *trp* and *inaC;trp* retinas. Furthermore, $\Delta[Ca^{2+}]_o$ in *inaC* was significantly larger than in WT at all light intensities.

$[Ca^{2+}]_o$ (Fig. 4 *A*) and at the maintained phase 60 s after light onset (Fig. 4 *B*) for the *trp, inaC;trp* double mutant, WT and *inaC* (as indicated).

At all light intensities both peak and maintained decrease in $[Ca^{2+}]_o$ were significantly larger in *inaC* relative to WT. In both *trp* and *inaC;trp* mutants, the

maintained component of $\Delta[Ca^{2+}]_o$ was virtually absent and also the transient phase was greatly diminished relative to WT or *inaC*. The transient phase of $\Delta[Ca^{2+}]_o$ at intense lights was significantly larger in *inaC;trp* relative to *trp* flies.

The Absence of Eye PKC Resulted in Enhanced Light-induced Increase in Cellular Ca^{2+}

To examine whether the enhanced decrease in $[Ca^{2+}]_o$ in *inaC* is due to enhanced Ca^{2+} influx into the photoreceptors, we measured the light-induced increase in cellular Ca^{2+} in WT and *inaC* flies using Ca Green-5N-loaded photoreceptors in isolated ommatidia. A detailed account of this technique is described by Peretz et al. (1994).

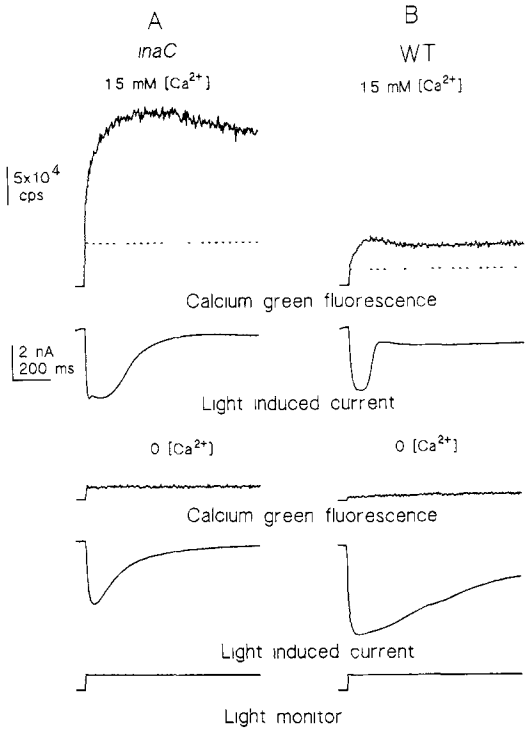

FIGURE 5. A comparison of the light-induced increase in cellular Ca^{2+} and LIC in wild-type and *inaC* photoreceptors. Simultaneous measurements of light-induced current and Ca Green-5N fluorescence in wild-type *Drosophila* photoreceptors voltage clamped at −50 mV (*A*) and in *inaC* (*B*) at 1.5 mM and zero external $[Ca^{2+}]_o$. (*Top*) Ca Green-5N fluorescence and LIC measured at 1.5 mM $[Ca^{2+}]_o$. The horizontal dotted line indicates the fluorescence level at the time of full opening of the shutter that controlled the exciting light. The background fluorescence (measured after removal of the ommatidium) was subtracted from all traces and from the values of the fluorescence in Table I. (*Bottom*) Ca Green-5N fluorescence and LIC measured at zero (~4 μM) external $[Ca^{2+}]$. (*Bottom line*) Photodiode current showing the output of a photodiode that monitored the excitation light. Calibration bar of the fluorescence intensity is expressed in counts per s (*cps*).

Fig. 5 shows simultaneous measurements of light-induced current (LIC) and Ca green 5-N fluorescence (the fluorescent Ca^{2+} signal) at −50 mV membrane potential in *inaC* (Fig. 5 *A*) and in WT (Fig. 5 *B*) bathed in Ringer solution containing 1.5 mM or 0 mM $[Ca^{2+}]$. The LIC in response to the intense excitation light in WT in 1.5 mM $[Ca^{2+}]_o$ was composed of a short (~6 ms) latency followed by a fast (~15 ms time to peak) increase in LIC which quickly declined towards baseline. A second phase of the

LIC followed the decline of the initial transient. This phase was composed of a small, delayed increase in LIC followed by a slow decline towards a small maintained component. The LIC of WT at zero $[Ca^{2+}]_o$ was significantly larger and slower relative to the LIC of WT at 1.5 $[Ca^{2+}]_o$. The LIC measured in *inaC* at 1.5 mM $[Ca^{2+}]_o$ had a different wave form: the initial transient phase showed a fast small component at the peak which was missing in the LIC of WT. Also the decline towards baseline was ~2.5 times slower relative to WT (see Smith et al., 1991; Hardie et al., 1993; Hardie and Minke, 1994 for details). The LIC of *inaC* at zero $[Ca^{2+}]_o$ was significantly smaller and declined much faster relative to the LIC measured at zero $[Ca^{2+}]_o$ in WT (see Hardie et al., 1993).

The opening time of the shutter (~6 ms) limited our ability to resolve the fluorescence during the latent period of the electrical response. We could only measure changes in fluorescence due to light-induced elevation of Ca^{2+} after the shutter fully opened. Before this time, the fluorescence results from autofluorescence and dye fluorescence due to resting Ca^{2+} plus any early light-induced changes in $[Ca^{2+}]$ (We call this initial fluorescence cellular background level). Our inability to detect any change in fluorescence after the shutter opened in the absence of cells, in unloaded cells, in *norpA* mutants (Bloomquist et al., 1988) which eliminate all responsivity to light (Pak et al., 1970) or in cells loaded with the Ca^{2+} insensitive dye fluorescein (see Peretz et al., 1994) indicates that the changes in fluorescence observed after the shutter opened represent a light induced elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) associated with the electrical response.

In the fluorescence traces of Fig. 5 at 1.5 mM $[Ca^{2+}]_o$, the fluorescence detected at the earliest time for which the shutter was fully open is marked by a dotted line. Once the shutter was open fully, the fluorescence continued to rise rapidly for an additional 10 ms, before the rate of rise slowed and the fluorescence reached a maximum level with a half rise time of ~0.1 s. The fluorescent Ca^{2+} signal declined slowly after reaching this maximum to a maintained high Ca^{2+} level which persisted indefinitely. The Ca^{2+} signal of *inaC* (Fig. 5 *A*) had a wave form similar to that of WT but on the average three times larger. When Ringer's solution without added Ca^{2+} was applied to the bathing solution (containing ~4 μM $[Ca^{2+}]_o$ as measured with Ca^{2+} selective electrode) the initial cellular background level was significantly reduced and the maximum further increase became very small and slow in both WT and *inaC* (*bottom two traces,* 0 $[Ca^{2+}]$ and Table I). Fig. 5 and Table I indicate that the fluorescent Ca^{2+} signal arise almost exclusively from Ca^{2+} influx (see Peretz et al., 1994, for details).

Table I compares the fluorescence measurements of WT and *inaC* ommatidia at −50 mV holding potential at 1.5 mM and zero $[Ca^{2+}]_o$, showing the average magnitude of the fluorescence observed at the earliest time for which the shutter was fully open (F) and the maximum further increase in fluorescence (ΔF) observed after the opening of the shutter (after subtraction of the fluorescence without the ommatidium). Table I shows that both the resting $[Ca^{2+}]$ and the light-induced increase in cellular $[Ca^{2+}]$ are significantly larger in *inaC* relative to WT. At zero $[Ca^{2+}]_o$, however, there is no significant difference between *inaC* and WT in either F or ΔF values.

DISCUSSION

The results presented here indicate that light induces a much larger increase in $[Ca^{2+}]_i$ in the photoreceptors of the eye-PKC mutant *inaC*, than in wild-type and that the large increase in $[Ca^{2+}]_i$ is due to an enhanced Ca^{2+} influx. The experiments also show that the *trp* mutation diminishes the light-evoked Ca^{2+} influx, consistent with earlier studies showing that the *trp* mutation diminishes a major fraction of the Ca^{2+} permeability of the light-sensitive channels (Hardie and Minke, 1992) and decreases the light-evoked increase in $[Ca^{2+}]_i$ (Peretz et al., 1994). The results furthermore show that the enhanced Ca^{2+} influx observed in the absence of eye-PKC is diminished in the double mutant *inaC;trp* indicating that the *trp*-dependent channels are required for both the normal and the enhanced Ca^{2+} influx.

TABLE I

Fluorescence Measurements of WT and inaC Ommatidia at −50 mV Holding Potential Using Ca Green-5N

	A		B	
	1.5 mM calcium		0 calcium	
	WT (n = 10)	*inaC* (n = 9)	WT (n = 4)	*inaC* (n = 4)
		$\times 10^5$ *cps*		
S_{max}	0.87 ± 0.11	2.55 ± 0.33	0.17 ± 0.05	0.22 ± 0.03
$\overline{F}$	0.39 ± 0.06*	0.93 ± 0.13*	0.07 ± 0.01	0.09 ± 0.02
$\overline{\Delta F}$	0.48 ± 0.06*	1.65 ± 0.28*	0.10 ± 0.05	0.14 ± 0.02

$S_{max} = \overline{F} + \overline{\Delta F}$

$\overline{F}$ is the average magnitude of the fluorescence at the earliest time for which the shutter was fully opened.

$\overline{\Delta F}$ is the average magnitude of the fluorescence after the shutter was fully opened and the maximum further increased.

There is a significant difference in $\overline{\Delta F}$ and $\overline{F}$ between WT and *inaC* cells at 1 5 mM calcium.

There is no significant difference in $\overline{\Delta F}$ and $\overline{F}$ at 0 calcium between WT and *inaC* cells.

*5% significance level in t test.

SEM, ± standard error of the mean.

Protein Kinase C Is Required for Controlling Ca^{2+} Influx and for Light Adaptation

A previous study on the PKC null mutant $inaC^{P209}$ suggested that the primary defect which may account for the pleiotropic manifestations of the *inaC* phenotype is the failure of individual quantum bumps to terminate normally. Assuming that Ca^{2+} release from the $InsP_3$-sensitive stores is required for excitation, possible underlying mechanisms for the slowly terminating bumps include the lack of negative feedback of Ca^{2+} on Ca^{2+} release from the stores (e.g., Payne et al., 1988) or defective Ca^{2+} sequestering mechanisms (Hardie et al., 1993). On this assumption PKC, presumably itself activated by a light-induced rise in cytosolic Ca^{2+} and production of diacylglycerol, is required for effective termination of the single photon events and the macroscopic light response by terminating the rise in cytosolic Ca^{2+} induced by absorption of each photon. The magnitude of the Ca^{2+} influx via the *trp*-dependent

channels during light depends on the duration of the quantum bumps. The enhanced Ca^{2+} influx and the resulting large increase in cellular Ca^{2+} in *inaC* during light are thus consistent with the greatly delayed closure of the *trp* dependent channels. Conversely, elimination of the *trp*-dependent channels by a mutation is expected to reduce both the normal Ca^{2+} influx of WT and the enhanced Ca^{2+} influx found in *inaC*. These expectations were fulfilled. The fact that in the double mutant *inaC;trp* the remaining Ca^{2+} signal was slightly enhanced relative to *trp* suggests that PKC also controls Ca^{2+} influx via *trp* independent pathway.

According to the capacitative Ca^{2+} entry model (Putney, 1990), depletion of the $InsP_3$-sensitive Ca^{2+} stores initiates Ca^{2+} influx into variety of cells. If a similar model applies to *Drosophila* photoreceptors, then the presumed large depletion of the Ca^{2+} stores in *inaC* after illumination should enhance Ca^{2+} influx in *inaC* relative to WT. The results of the present study are consistent with such hypothesis. In particular, at zero external [Ca^{2+}] the LIC in *inaC* was much smaller relative to WT under the same conditions while the opposite behavior was observed at 1.5 mM external Ca^{2+} (Fig. 5).

It is well established that light adaptation in invertebrate photoreceptors is mediated by an increase in cellular Ca^{2+} (Lisman and Brown, 1972), with a major candidate mechanism being the negative feedback of Ca^{2+} on Ca^{2+} release from the $InsP_3$-sensitive Ca^{2+} stores (Payne et al., 1988). In *inaC* under physiological conditions, light adaptation is virtually absent (Hardie et al., 1993). The present study reveals that in spite of the absence of light adaptation in *inaC,* light induces an abnormally high increase in cytosolic Ca^{2+}, thus suggesting that PKC is a possible link between the rise in cytosolic Ca^{2+} and light adaptation.

The Use of Ca^{2+}-selective Electrodes to Monitor Ca^{2+} Influx

The use of Ca^{2+}-selective microelectrodes to monitor [Ca^{2+}] in the extracellular space, has been established as a sensitive and reliable measure of changes in $[Ca]_o$ in insect retina (Minke and Tsacopoulos, 1986; Ziegler and Walz, 1989; Sandler and Kirschfeld, 1988, 1991, 1992; Rom-Glas et al., 1992). A major advantage of the technique is the ability to monitor [Ca^{2+}] changes in the intact retina under physiological conditions. Control experiments and calculations (see Appendix) suggest that the reduction in $[Ca^{2+}]_o$ reflects Ca^{2+} influx. Firstly, it has been shown by several detailed studies that the possible existence of a dead space of destroyed tissue around the 2–3-μm wide electrode tip has a negligible effect on extracellular ion concentration measurements as long as the dead space is small with respect to the intact extracellular space in communication with it (Lux and Neher, 1973; Sandler and Kirschfeld, 1988; summarized by Coles, 1986). Since the Lucifer yellow CH staining method showed negligible damage caused by the recording electrodes (see Materials and Methods), we conclude that there is a negligible error in our $\Delta[Ca^{2+}]_o$ measurements due to damaged tissue. Secondly, to show that the light-evoked influx of Ca^{2+} into the photoreceptors is the likely cause for most of the reduction in $[Ca^{2+}]_o$, we attempted to estimate the amount of Ca^{2+} entering a photoreceptor during light by calculating the fraction of the light-induced inward current (LIC) carried by Ca^{2+} and compared this to the amount of Ca^{2+} entering the cell as calculated from the decrease in $[Ca^{2+}]_o$ measured with Ca^{2+}-selective electrodes (see

Appendix). In WT the calculations based on LIC in response to 5 s of log-3 light intensity showed that 0.93×10^9 Ca^{2+} ions enter the cell while only 1.53×10^7 Ca^{2+} ions (e.g., ~60 times less) enter the cell according to calculations based on the decrease in $[Ca^{2+}]_o$. In *trp* in contrast, the calculations based on LIC in response to the same stimulus showed that 1.78×10^7 ions enter the cell via the LIC while 0.84×10^7 Ca^{2+} ions enter the cell according to calculations based on the decrease in $[Ca^{2+}]_o$.

A likely explanation for an *overestimation* of Ca^{2+} influx when calculated from the LIC in WT and *inaC* is that the predicted large influx of Ca^{2+} can be expected to temporarily reduce the driving force (EMF) for Ca^{2+} entry thereby reducing the overall flux relative to theoretical calculations assuming constant EMF. On the other hand, *underestimation* of Ca^{2+} influx based on $\Delta[Ca^{2+}]_o$ is expected because a significant fraction of Ca^{2+} entering the photoreceptors during light is rapidly extruded by the Na^+-Ca^{2+} exchanger. In the honeybee drone, the amount of Ca^{2+} extruded by the Na^+-Ca^{2+} exchanger is initially larger than that entering the cell (Minke and Tsacopoulos, 1986; Ziegler and Walz, 1989). However, unlike the retinal slices of the honeybee drone, in which the Na^+-Ca^{2+} exchanger can be blocked by perfusion with 0 Na^+ Ringer's solution during $[Ca^{2+}]_o$ measurements, this experimental procedure is not presently feasible in *Drosophila* eye. Therefore, we cannot give a quantitative estimate for the amount of Ca^{2+} extruded by the Na^+-Ca^{2+} exchanger in *Drosophila*. Measurements of cellular Ca^{2+} in the pigment cells of *Drosophila* as performed in the honeybee drone (Coles and Orkand, 1984) are also not feasible. Ca^{2+} movement into or out of the pigment cells surrounding the ommatidia may influence $\Delta[Ca^{2+}]_o$ measurement by some unknown factor. By analogy to the drone retina we assume that these effects are small.

The above considerations indicate that light-induced Ca^{2+} influx into the photoreceptors is more than sufficient to account for the measured decrease in extracellular $[Ca^{2+}]$ and suggest that the increase in $[Ca^{2+}]_i$ (of 31.7 μM, in WT at log-3 stimulus) calculated from the decrease in $[Ca^{2+}]_o$ is underestimated because of Ca^{2+} extrusion by the Na^+-Ca^{2+} exchanger (see Appendix). In the *trp* mutant, the calculated influx based on $\Delta[Ca^{2+}]_o$ fits the calculated Ca^{2+} influx based on the LIC. A likely explanation is that the diminished Ca^{2+} influx in *trp* reduced only little the EMF for Ca^{2+} entry. Since ~99% of Ca^{2+} ions which enter cells are probably quickly buffered (e.g., Neher and Augustine, 1992) the free Ca^{2+} concentration available for extrusion by the Na^+-Ca^{2+} exchanger can be expected to be minimal in *trp* under physiological conditions. In contrast, in WT and *inaC,* during intense light, the amount of Ca^{2+} influx is so large that even 1% of the influx is expected to raise cellular free Ca^{2+} to a level which activates the Na^+-Ca^{2+} exchanger to a high activity. Therefore, we suggest that the decrease in $[Ca^{2+}]_o$ is likely to reflect a lower limit for Ca^{2+} influx into the cell in WT and inaC, (but not necessarily in *trp*) and that differences in $\Delta[Ca^{2+}]_o$ observed between WT, *inaC* and *trp* are likely to reflect differences in Ca^{2+} influx. This suggestion was supported by direct measurements of light-induced increase in cellular Ca^{2+} using Ca^{2+} indicator fluorescence in WT, *inaC* (Fig. 5) and *trp* flies (Peretz et al., 1994).

Figs. 1 and 2 show that $\Delta[Ca^{2+}]_o$ has a complex wave form. Understanding the

complex waveforms of $\Delta[Ca^{2+}]_o$ at different light intensities in WT, *inaC* and *trp* requires further investigation which is outside the scope of the present study.

CONCLUSIONS

The present results are consistent with the hypothesis that TRP is a light-activated Ca^{2+} channel, while eye-PKC controls Ca^{2+} influx via TRP-dependent channels. These conclusions, derived from measurements of the decrease in $[Ca^{2+}]_o$ using Ca^{2+}-electrodes in the intact preparation support earlier electrophysiological studies of the ionic permeability of the light-activated conductance (Hardie and Minke, 1992; Hardie et al., 1993) and have recently been confirmed more directly by measuring the increase in cellular Ca^{2+} in WT, *trp* and *inaC* using fluorescence of the Ca^{2+} indicator Ca Green-5N in internally dialyzed photoreceptors (Peretz et al., 1994; and Fig. 5).

APPENDIX

Estimation of Light-induced Ca^{2+} Influx from Measurements of LIC and $\Delta[Ca^{2+}]_o$

The changes in extracellular $[Ca^{2+}]$ during and after light depend on at least four factors: (*a*) Influx of Ca^{2+} into the photoreceptors via the light sensitive channels. (*b*) Efflux of Ca^{2+} from the photoreceptors via Na^+-Ca^{2+} exchanger. (*c*) Influx or efflux of Ca^{2+} into the pigment (glia) cells. (*d*) Changes in the volume of the extracellular space.

As previously argued for the case of bee photoreceptors (Minke and Tsacopoulous, 1986) the most likely cause for a decrease in $[Ca^{2+}]_o$ is the light-induced influx of Ca^{2+} into the photoreceptors. To see whether this influx is sufficient to account for the observed decrease in $[Ca^{2+}]_o$ in *Drosophila,* we attempted to estimate the amount of Ca^{2+} entering a photoreceptor during light as follows, by calculating the fraction of the light-induced inward current carried by Ca^{2+}.

The dependence of the reversal potential (E_{rev}) of the LIC on $[Ca^{2+}]_o$ has been used to estimate the permeability of Ca^{2+} relative to other permeable ions during the LIC. This ratio was estimated as 40:8:1 for Ca^{2+}, Mg^{2+} and Na^+ (Hardie and Minke, 1992). Using the Goldman, Hodgkin, and Katz (GHK) constant current equation (e.g., Hille, 1992) and the driving force for each of the above ions, it can be estimated that Ca^{2+} carries 35–40% of the light induced current in our Ringer over the physiological voltage range. The mean intracellularly recorded voltage during a 5-s response to the Xenon light attenuated by 3-log units is ~ -35 mV (5 s is the time to peak of $\Delta[Ca^{2+}]_o$ in WT in response to the corresponding stimulus, Fig. 2). The mean voltage of the receptor potential was calculated by sampling the membrane potential during light from the time of detectable departure of the receptor potential from the resting potential value during 5 s in intervals of 1 ms. The sum of the sampled voltage values was divided by the number of the sampling points ($5 \cdot 10^3$). The very short initial transient (~ 60 ms duration) contributed only 4 mV to the mean voltage. The integral of the LIC measured by whole-cell recording at -35 mV is 0.88×10^{-9} C (there is $\sim 30\%$ variability in the amplitude of the LIC in different cells). Assuming that 35% of the LIC is carried by Ca^{2+} it follows that 0.31×10^{-9}

C, corresponding to 1.55×10^{-15} mole Ca^{2+} or 0.93×10^{9} Ca^{2+} ions are estimated to enter the cell during the 5-s stimulus. Note, however, that this calculation necessarily assumes the standard assumptions of GHK theory, most notably the independent mobility of ions, and furthermore that the predicted large flux of Ca^{2+} can be expected to temporarily reduce the EMF thereby reducing the overall flux (see Discussion).

Unlike WT, the LIC in *trp* declines exponentially to baseline within 5 s during the corresponding light intensity. In intracellular recordings, the mean voltage during this time is ~ -40 mV. The peak response amplitude when measured with microelectrodes was found to be relatively small in *trp*. The total charge carrying the LIC at this holding potential is 0.59×10^{-10} C. The permeability of Ca^{2+} relative to other permeating ions in the *trp* mutant was estimated as 3.5:1.9:1 for Ca^{2+}, Mg^{2+}, and Na^{+} (Hardie and Minke, 1992). Under our experimental conditions Ca^{2+} can be expected to carry 10% of the total LIC in *trp* flies. Accordingly, 0.59×10^{-11} C corresponding to 2.95×10^{-17} mol Ca^{2+} or 1.78×10^{7} Ca^{2+} ions can be expected to enter the cells in *trp* during 5 s of the log-3 stimulus.

The above estimates of Ca^{2+} entering the cells can be compared to the amount of Ca^{2+} entering the cells calculated from the decrease in extracellular $[Ca^{2+}]$ measured with Ca^{2+} selective electrodes. In WT retina, the peak decrease in $[Ca^{2+}]_o$ to log-3 stimulus is 0.2 mM (Fig. 5 *A*). The averaged volume of R1-6 photoreceptor is 0.8×10^{-12} liter (assuming 3.5 μm cell diameter, 83 μm cell length and cylindrical shape, Hardie, 1985). The volume of the photoreceptors has been estimated as 6.3 times larger than the volume of the extracellular space in fly retina (Sandler and Kirschfeld, 1988). Using the above data it follows that the average concentration increase of cellular Ca^{2+}, 5 s after the onset of log-3 stimulus is 31.7 μM, assuming that the reduction in $[Ca^{2+}]_o$ is only due to Ca^{2+} influx into the photoreceptors. This increase in cellular $[Ca^{2+}]$ corresponds to 2.54×10^{-17} mol or 1.53×10^{7} Ca^{2+} ions. This value is smaller by a factor of ~ 60 relative to that calculated from the LIC.

The peak decrease in $[Ca^{2+}]_o$ in the *trp* retina to log-3 stimulus is 0.11 μM which corresponds to 17.5 μM internal $[Ca^{2+}]$ or 1.4×10^{-17} mol Ca^{2+} or 0.84×10^{7} Ca^{2+} ions entering a cell, assuming the same assumptions used for WT. This value is similar to that calculated from LIC measured in the *trp* fly.

Shrinkage of the extracellular space due to increase in volume of the photoreceptors during intense light causes up to 30% reduction in the volume of the extracellular space of the drone retina (Orkand et al., 1984; Ziegler and Walz, 1989). In the drone the volume of the extracellular space is estimated as only 4.8% the volume of the photoreceptors (Coles and Tsacoupolos, 1979). In the fly, in contrast, the volume of the extracellular space is estimated as 16% the volume of the photoreceptors. This makes fly extracellular space less sensitive to changes in the volume of the photoreceptors. In addition, no volume change was found in *Drosophila* isolated ommatidia during intense lights when measured by the fluorescence of fluorescein-loaded photoreceptors (Peretz et al., 1994).

The above considerations support our conclusion that in *Drosophila*, like in the bee photoreceptors, the decrease in $[Ca^{2+}]_o$ reflects mainly the light-induced Ca^{2+} influx. The series of approximations and assumptions that needed to be made to perform the calculations, most likely affect the calculated values of Ca^{2+} influx but do not invalidate the conclusion.

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