Photolysis of Caged Ca^{2+} Facilitates and Inactivates but Does Not Directly Excite Light-Sensitive Channels in *Drosophila* Photoreceptors

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Substantial evidence implicates the phosphoinositide cascade in invertebrate phototransduction, but the final pathway of excitation remains obscure. In order to test the hypothesis that Ca^{2+} is the excitatory messenger rapid concentration jumps of cytosolic Ca^{2+} were achieved in dissociated *Drosophila* photoreceptors by flash photolysis of the caged Ca^{2+} compounds DM-nitrophen and nitr-5. Both compounds were introduced via patch pipettes used to record whole-cell currents. Calibrations using INDO-1 and Mag-INDO-1 indicated that photolysis of DM-nitrophen (5 mM loaded with 4 mM Ca^{2+}), raised Ca, to ca. 20–50 μM , and nitr-5 (same loading) to ca. 1–2 μM . In mutants lacking light responses (*ora*, lacking rhodopsin; *norpA*, lacking phospholipase C; *trp*, which is inactivated by conditioning lights), the only current evoked by photolysis of DM-nitrophen was a small inward current with no detectable latency. This current did not reverse at +80 mV and was blocked by substitution of external Na^+ for Li^+ , suggesting it represents activation of an electrogenic $\text{Na}^+/\text{Ca}^{2+}$ exchanger. A similar current was also the only current elicited by caged Ca^{2+} during the 5 msec latent period in wild type (WT) photoreceptors. To investigate possible modulatory effects of caged Ca^{2+} on the light-activated conductance, cells were first stimulated with a saturating light stimulus, itself incapable of releasing significant Ca^{2+} , and then the photolytic flash was discharged during the response. During the rising phase of the response, photolysis of DM-nitrophen (but not nitr-5) induced a pronounced facilitation in WT photoreceptors. When photolysed during the plateau phase both DM-nitrophen and nitr-5 induced a rapid inactivation of the light-induced current. By contrast, in *trp* photoreceptors, which lack one class of Ca^{2+} permeable light-sensitive channel, photolysis of DM-nitrophen induced a significant facilitation during the falling phase of the response, but during the rising phase photolysis significantly depressed the overall response. In conclusion, caged Ca^{2+} failed to activate any channels in *Drosophila* photoreceptors but profoundly affected the light-dependent channels once they have been activated.

[Key words: photoreceptors, inositol phosphates, calcium, *Drosophila*, sodium-calcium exchange, phototransduction, caged calcium, DM-nitrophen, INDO-1]

Phototransduction in invertebrate photoreceptors is believed to be mediated, at least in part, via the ubiquitous phosphoinositide signalling cascade (Brown et al., 1984; Fein et al., 1984; Bloomquist et al., 1988; reviews: Payne et al., 1988; Nagy, 1991; Berridge, 1993). While light-dependent GTP-ase activity (Blumenfeld et al., 1985), phospholipase C activity (Devary et al., 1987), and InsP_3 -induced Ca^{2+} release (Payne et al., 1988) have all been demonstrated, the mechanism by which cation-selective channels are activated in the plasma membrane remains obscure. In *Drosophila* photoreceptors the light sensitive channels are highly permeable to Ca^{2+} . Since the permeability to Ca^{2+} is greatly reduced in null mutants of the *trp* gene (Cosens and Manning, 1969; Hardie and Minke, 1992, 1993), and since the sequence of the *trp* gene product (Montell & Rubin, 1989) shows homologies with vertebrate voltage-gated Ca^{2+} channels (Phillips et al., 1992), it has been proposed that *trp* encodes one class of light-sensitive channel (Hardie and Minke, 1992; Phillips et al., 1992), whilst a second class of light-sensitive channel with lower permeability to Ca^{2+} is still present in the *trp* mutant. Since the light response mediated by the channels in the *trp* mutant decays rapidly to baseline (Cosens and Manning, 1969; Minke, 1982), maintained activity of the channels remaining in the mutant apparently requires influx of Ca^{2+} via the *trp*-dependent channels. This has led to the suggestion that the non-*trp*-dependent class of channel may be activated directly or via an intermediate by Ca^{2+} (Hardie and Minke, 1992, 1993; Minke and Selinger, 1992). Substantial evidence also supports an excitatory role for Ca^{2+} in *Limulus* ventral photoreceptors. Thus prolonged exposure to Ca^{2+} -free Ringer's (Bolsover and Brown, 1985) or injection of potent Ca^{2+} buffers such as BAPTA or dibromo-BAPTA (Frank and Fein, 1991; Shin et al., 1993) severely reduces sensitivity. Furthermore, injection of Ca^{2+} activates an inward conductance with similar reversal potential to the light-activated conductance (Payne and Fein, 1986; Shin et al., 1993). Nevertheless, the hypothesis that Ca^{2+} is the final messenger of excitation is still controversial, since measurements with Ca^{2+} indicator dyes suggest that the light-induced rise in cytosolic Ca^{2+} lags the electrophysiological response (Stieve and Benner, 1992) and because no evidence exists showing that Ca^{2+} is capable of directly gating channels in the membrane. Indeed the only substance reported to activate channels in excised patches containing light-sensitive channels is cGMP (Bacigalupo et al., 1991). This has led on the one hand to models

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suggesting multiple pathways of excitation (e.g., Frank and Fein, 1991; Nagy, 1991; Nagy et al., 1993) and on the other to the suggestion that Ca^{2+} is an intermediate messenger of excitation required for raising levels of cGMP (Shin et al., 1993).

In *Drosophila*, there are no reports of excised patch recordings of light-sensitive channels, but an alternative approach to testing the effects of putative messenger substances on channels is to supply candidates internally. A powerful method for controlled and rapid application is provided by flash photolysis of photolabile caged compounds (Kaplan and Ellis-Davies, 1988; reviews, Kaplan, 1990; Adams and Tsien, 1993). In *Drosophila* the ability to make whole-cell recordings (Hardie, 1991a,b; Ranganathan et al., 1991) should allow internal perfusion of known concentrations of such compounds, while the availability of mutants with no response to light (review, Pak, 1991) should allow the effects of the released compounds to be investigated without contamination by the light-induced current itself. In this study the effects of the release of Ca^{2+} from two different photolabile Ca^{2+} chelators, DM-nitrophen and nitr-5, have been investigated in both wild type and mutant photoreceptors. The results indicate that photolytically released Ca^{2+} can act to both facilitate and inactivate the channels once the light response has been initiated, but appears to be insufficient to gate the light-dependent channels by itself.

Materials and Methods

Flies. The "wild type" (WT) strain was white-eyed (*w*) Oregon R (OR) which lacks all retinal screening pigments but has otherwise normal physiology. The following mutants (all on a *w* OR background) were also used: *trp^{CM}* (Cosens and Manning, 1969; Montell and Rubin, 1989) lacking a light-dependent Ca^{2+} channel (Hardie and Minke, 1992); a strain lacking the rhodopsin gene *ninaE* (O'Tousa et al., 1985; Zuker et al., 1985); *ninaE^{ora} es/1M6* (referred to subsequently as *ora*); and *norpA²⁴*, a null allele of the phospholipase C gene (Bloomquist et al., 1988). Flies were raised at 25°C. Late stage pupae (p15) staged according to Bainbridge and Bownes (1981) or newly eclosed adults (<4 hr post-eclosion) were used.

Preparation. Whole-cell recordings were performed on photoreceptors from dissociated *Drosophila* ommatidia prepared as previously described (Hardie, 1991a,b; Hardie and Minke, 1992, 1994a,b). Ommatidia were allowed to settle in a small chamber the bottom of which was formed by a coverslip and viewed with a 40× oil immersion objective (Nikon CF-fluor, NA 1.30) on the stage of an inverted Nikon Diaphot microscope. Stimulation was provided via the objective using the fluorescence epiillumination system of the microscope with a dichroic mirror reflecting light below 380 nm (Nikon DM 380). Light was delivered to the fluorescence port via a forked quartz optic light guide and an adaptor lens (PTI instruments, South Brunswick, NJ). One arm of the light guide received light from an XF-10 flash lamp (Hi-Tech Scientific, UK) capable of discharging up to 385 V (generating 244 Joules) within ca. 2 msec. The other arm was connected to the output of an illumination system consisting of a 75 W Xe arc lamp, monochromator (PTI), and a Uniblitz shutter with rise time 1.8 msec (Vincent Associates, Rochester, NY). In the majority of experiments a standard submaximal flash (300 V, 104 Joules) was delivered 2 min after establishing the whole-cell configuration. Prior to the flash the series resistance was constantly monitored and if necessary gentle positive pressure applied intermittently to maintain a low series resistance and to encourage perfusion of the cell with the electrode contents.

Clamp quality. Series resistances were typically in the range 10–25 MΩ, series compensation typically in the range 70–80%. Although these conditions provide adequate voltage-clamp control for currents of up to ca. 2 nA, some distortion of the larger currents generated in this study must be expected.

Solutions. Bath solution contained (in mM) 120 NaCl, 5 KCl, 10 *N*-Tris-(hydroxymethyl)-methyl-2-amino-ethanesulphonic acid (TES), 8 MgSO₄, 30 sucrose, 2 proline, and unless otherwise stated 1.5 mM CaCl₂. DM-Nitrophen (tetrasodium salt, Calbiochem, San Diego, CA) was included in the electrode solutions which were based on either: 130

K gluconate 20 TES, or 110 CsCl 15 tetra-ethyl ammonium (TEA) Cl and 20 TES. DM-Nitrophen is based on the divalent cation chelator EDTA and like EDTA also binds (and releases) Mg²⁺ ions (Kaplan and Ellis-Davies, 1988). Mg²⁺ was thus omitted altogether, and in most cases 4 mM K₂ATP was also present to chelate any residual Mg²⁺. For all experiments described DM-nitrophen was used at a concentration of 5 mM and loaded with 4 mM Ca²⁺ (80% loaded); however, a number of trials performed under different loading conditions (5 mM, 50% and 60% loaded; 10 mM 80% loaded) produced qualitatively similar results in both WT and mutant flies. Two kinds of controls were performed: for testing the effect in cells which did not respond to light (*ora*, *norpA*, and inactivated *trp*) the same concentration of DM-nitrophen was used but Ca²⁺ was either omitted or replaced by 4 mM Mg²⁺. Since intracellular Ca²⁺ buffering profoundly influences the light response (e.g., Hardie et al., 1993), for controls in experiments involving the light response, an additional control solution was used in which the Ca/DM-nitrophen was replaced by 5 mM EDTA/3 mM Ca²⁺ which should provide roughly comparable Ca²⁺ and Mg²⁺ buffering conditions. For experiments with nitr-5, the same loading conditions were used (5 mM nitr-5 : 4 mM CaCl₂), but since nitr-5 has no significant affinity for Mg²⁺ and little pH sensitivity, normal Mg²⁺ concentrations were used (2 mM MgSO₄, 4 mM MgATP) and only 10 mM TES. All solutions were buffered to pH 7.15.

Calibration. In order to estimate the amount of Ca²⁺ released by the caged compounds a number of *in vitro* and *in vivo* calibration trials were performed. In these experiments, in addition to the same concentration of caged Ca²⁺ (5 mM, 80% Ca²⁺ loading), the electrode solution also contained 100 μM of the dual emission ratiometric Ca²⁺ indicator INDO-1 or Mag-INDO-1 (Calbiochem). *In vitro* trials were performed with drops of solutions under glass coverslips in the recording chamber. *In vivo* trials were performed in "blind" photoreceptors (i.e., *ora*, *norpA*, and inactivated *trp*) all of which yielded similar results. Numerous controls confirmed that there is no light-induced Ca²⁺ rise in any of these mutants (data not shown). After allowing the standard time of 2 min for equilibration of the dye and caged compounds, the Ca²⁺ indicator dye fluorescence was measured (PTI fluorescence system: photon counting mode, ratio of fluorescence at 405 and 480 nm, after subtraction of background measured from the same cell before establishing the whole-cell configuration) during a 200 msec test pulse of 360 nm light from the 75 W Xe lamp and monochromator. Subsequently the Hi-Tech Xe flash lamp was discharged and the fluorescence measured again. The photomultiplier tubes were protected from the Xe flash discharge by a manually controlled shutter, and consequently the second Ca²⁺ measurement was made ca. 1/2 sec after the Xe flash. It is therefore possible that a larger transient rise in Ca²⁺ is elicited as reported for DM-nitrophen under some loading conditions (Zucker, 1993).

Fluorescence ratios (*R*) were converted into free Ca²⁺ concentrations using the equation (Grynkiewicz et al., 1985) $[\text{Ca}] = K_d^* \cdot (R - R_{\min}) / (R_{\max} - R)$, where K_d^* , the effective dissociation constant (K_d) for the dye, $= K_d \cdot (F_0/F_s)$, where F_0/F_s is the ratio of the fluorescence at 480 nm in the presence (F_s) and the absence (F_0) of saturating Ca²⁺. For INDO-1, $R_{\min}$ (minimum fluorescence ratio in low Ca²⁺) was determined with solutions containing 10 mM EGTA, 40 mM KOH, 90 mM K gluconate, 20 mM TES, and 5 mM caged compound. For $R_{\max}$ (maximum fluorescence ratio at saturating Ca²⁺ concentrations) the solution contained 15 mM CaCl₂, 110 K gluconate, 20 TES, and 5 mM caged compound. The effective K_d^* for INDO-1 was determined using a solution containing 5 mM EGTA and 3.5 mM Ca²⁺ which should provide a free Ca²⁺ concentration of 470 nM. The value obtained for K_d^* (1.3 μM) implies a K_d for INDO-1 of 250 nM assuming F_0/F_s values determined *in vitro* using the same solutions. Mag-INDO-1 is a derivative of INDO-1 which binds Mg²⁺, but in the absence of Mg²⁺ can be used as a Ca²⁺ indicator with much lower affinity than INDO-1. For calibration of Mag-INDO-1, $R_{\max}$ was determined as above, $R_{\min}$ was simply taken as the initial ratio before photolysis and effective K_d^* ($K_d^* = 310 \mu\text{M}$) was estimated using a solution containing 10 mM nitrilotriacetic acid and 2.5 mM Ca²⁺ (calculated free Ca²⁺ 50 μM). Examples of *in vivo* signals recorded following flashes with both DM nitrophen and nitr-5 in the cell are shown in Figure 1. The 200 msec measuring light (at 360 nm) was identical to that used to provide the saturating stimulus in two pulse experiments described below. This stimulus alone generates no detectable increase in Ca²⁺ with nitr-5 and only a relatively small increase (ca. 200–300 nM) in cells loaded with Ca/DM-nitrophen. Photolysis of nitr-5 resulted in a concentration jump from ca. 500 nM to 1–2 μM (mean: *in vivo*, from 492 ± 131 nM to 936 ± 187 nM, $n = 5$; *in vitro*, from 537 ± 65

nM to 1885 ± 683 nM, $n = 5$). The values reached with DM-nitrophen were typically near saturation when using INDO-1 (*in vivo*: greater than $5\text{--}10$ μM), while calibration trials with Mag-INDO-1 (with solutions containing no Mg^{2+}) indicated values of $20\text{--}50$ μM (mean: 26 ± 15 μM , $n = 6$). These concentration jumps are similar to those previously reported in the literature using similar loading conditions (review: Adams and Tsien 1993). Note, however, that no attempt was made to correct for possible influence of caged compounds on the K_d^* of the Ca^{2+} indicator dyes. If, as reported for fluo-3 (Zucker, 1993), K_d^* is shifted in the presence of caged compounds then these values may be in error by a factor of 2 or 3.

Results

Effects of caged Ca^{2+} in *ora*, *norpA*, and *trp*

A complication with using caged compounds in photoreceptors is that the flash used to release the caged compounds is sufficient by itself to generate saturating light-induced currents from which the photoreceptors never recover. Therefore for the first series of experiments the effects of caged Ca^{2+} were explored in photoreceptors of mutants lacking all response to light. These included: a severe *ora* allele which lacks the rhodopsin of R1-6 cells (O'Tousa et al., 1985) and *norpA* which lacks phospholipase C (Bloomquist et al., 1988). In addition the effects were tested in the *trp* mutant. In this mutant following prolonged steps of moderate to bright light intensities, the response decays to absolute baseline (Cosens and Manning, 1969; Minke, 1985; Hardie and Minke, 1992). The photoreceptor is subsequently refractory to additional light flashes for at least 30 sec and under whole-cell recording conditions often indefinitely.

As expected, in most cases the UV flash by itself generates no measurable current in *ora* mutants (Fig. 2*a*) when no caged Ca^{2+} was included in the electrode, or when the DM-nitrophen was loaded with Mg^{2+} instead of Ca^{2+} . This control also defines the flash artefact which is usually complete within ca. 200 μsec . A few *ora* photoreceptors were encountered which did give a small response to the UV flash indicating that in some cells a small amount of visual pigment is indeed produced, and also confirming that the remainder of the phototransduction cascade is intact in this mutant. In *norpA*, or inactivated *trp* photoreceptors, the UV flash never induces any maintained inward current; however, it does generate a substantial biphasic transient current with no measurable latency. This signal represents the charge movement due to conformational change of rhodopsin molecules within the microvillar membrane, that is, the voltage-clamped manifestation of the early receptor potential (ERP), subsequently referred to as early receptor current or ERC. As recently reported for the ERP in *Calliphora* (Gagne et al., 1989) the ERC is biphasic; however, the time course is much faster than the ERP since the response is no longer limited by the membrane time constant. Under voltage clamp the ERC consists of a rapid inward component, peaking in ca. 0.5 msec and which is probably limited by the clamp time constant, and a slower outward component peaking ca. 2 msec after the flash. The inward component is always larger, but the relative amplitude of the outward component increases with membrane depolarization.

When the electrode contained 5 mM DM-nitrophen loaded with 4 mM Ca^{2+} (Fig. 3), the UV flash consistently induces a small inward current in all three "blind" mutants (*ora*, *norpA*, and inactivated *trp*). As is most clearly seen in *ora*, this signal has no detectable latency (<0.5 msec). Its time course in fact closely follows that of the cumulative energy in the Xe flash and may be limited by the kinetics of Ca^{2+} release under these con-

Figure 1. Calibration of DM-nitrophen and nitr-5. Simultaneous recordings of membrane current (upper traces, cells clamped at -50 mV) and Ca^{2+} concentration measured using the Ca^{2+} indicator dyes INDO-1 and Mag-INDO-1 (100 μM). *a*, *norpA* photoreceptor loaded with INDO-1, 5 mM DM-nitrophen, and 4 mM Ca^{2+} . Prior to the photolytic flash $[\text{Ca}]$ is below 1 μM , it increases only slightly during the 200 msec 360 nm light used to excite the INDO-1 fluorescence. After the flash $[\text{Ca}^{2+}]$ has risen to ca. 10 μM , the high level of noise however, indicates that the INDO-1 is approaching saturation. The flash also induces a slowly decaying inward current which is attributed to the electrogenic $\text{Na}^+/\text{Ca}^{2+}$ exchanger. At this sampling frequency (100 Hz), the early receptor current (see Fig. 2) is not resolved. *b*, In an *ora* photoreceptor, loaded with the lower affinity Ca^{2+} indicator Mag-INDO-1, photolysis of the Ca/DM-nitrophen releases an estimated 20 μM free Ca^{2+} . The concentration before the flash is not reliably measured with this indicator. *c*, *trp* photoreceptor after inactivation by prior illumination, loaded with 5 mM nitr-5, 4 mM Ca^{2+} and INDO-1. An identical Xe flash increases $[\text{Ca}^{2+}]$ from ca. 350 nM to 800 nM. A very small inward current is still detectable with nitr-5 but only ca. 10% of that seen with DM-nitrophen.

Figure 2. Control responses to Xe flashes in photoreceptors of "blind" mutants *a*, In *ora*: Xe flash delivered to a p15 photoreceptor with an electrode containing 110 mM CsCl, 15 TEACl, 20 TES, 5 mM DM-nitrophen, 4 mM Mg^{2+} , and no Ca^{2+} . Apart from an electrical artefact no response is detected at either -50 mV or $+80$ mV. *b*, In *norpA* (adult) the Xe flash evokes a prominent biphasic response which is complete within ca. 3 msec. This is the voltage-clamped manifestation of the early receptor potential. The relative contribution of the inward component is reduced on depolarization to $+50$ mV.

ditions. At -50 mV the amplitude of this inward current is fairly consistent from cell to cell, averaging $46 \text{ pA} \pm 19 \text{ pA}$ ($n = 12$), or $1.85 \pm 0.54 \text{ pA/pF}$ after normalization for cell capacitance, during one experimental period of 2 weeks. Similar sized responses were obtained using electrode solutions based on either K gluconate or Cs/TEA Cl. Similar currents were observed both in Ringer containing physiological (1.5 mM) levels of Ca^{2+} and also in nominally Ca^{2+} free Ringer's; however, in Ca^{2+} free Ringer's the current was significantly larger ($2.2 \pm 0.3 \text{ pA/pF}$). On a slower time base the inward current decays to baseline over a time course of several seconds (Fig. 3c). Subsequent flashes delivered at one or two minute intervals induced similar size responses (data not shown). The size of the current was clearly intensity dependent and at later stages of this study,

Figure 3. Effects of DM-nitrophen loaded with Ca^{2+} . *a* and *b*, in both *ora* and inactivated *trp* a 300 V Xe flash evokes a rapid inward current of ca. 50 pA in cells loaded with 5 mM DM-nitrophen and 4 mM Ca^{2+} . The stimulus monitor shows the time course of the Xe flash, and the dotted line the cumulative energy of the flash normalized to the amplitude of the inward current. In *trp* (adult) the inward current is superimposed upon a prominent ERC. At $+80$ mV the response is much reduced but still inward. *c*, Time course of the flash induced inward current on a slower time scale (*ora* p15).

after installation of a replacement Xe bulb, somewhat larger responses were consistently observed under otherwise similar conditions (e.g., 76 pA , or $2.9 \pm 1.3 \text{ pA/pF}$, $n = 9$ in Ca^{2+} free Ringer's).

At first sight this signal might be interpreted as the activation of a Ca -activated inward conductance as has been suggested to underlie at least part of the light-induced current (Nagy, 1991; Hardie and Minke, 1993). However, apart from its small size (ca. 0.2% of the saturated LIC), a number of properties of this current make such an interpretation unlikely. Firstly, there is no detectable channel noise associated with this signal, although recording conditions were often such that noise from channels

of ca. 0.5–1 pS or more would have been reliably detected. Secondly, the LIC reverses at around +15 mV (Hardie, 1991a; Hardie and Minke, 1992); however, it was not possible to reverse the Ca-activated current at depolarized voltages (Fig. 3*a,b*). In order to explore the current–voltage relationship of this current in more detail, rapid ramps of voltage were applied immediately after activation during the first 100 msec of the response, that is, before any significant decay of the response has occurred (Fig. 4*a*). The current is still inward at +80 mV and there is no indication of the marked outward rectification which is characteristic of both suspected components of the light dependent conductance (Hardie, 1991a; Hardie and Minke, 1992). Instead the current shows an approximately exponential inward rectification with an e -fold increase for ca. 70 mV hyperpolarization.

An alternative explanation for a Ca-activated inward current with such properties would be activation of an electrogenic $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Although this is capable of operating in reverse, simply increasing Ca^{2+} on the inside of the membrane can only produce an inward current, and an exponential I – V relationship with a similar voltage dependency is well established in other systems (Kimura et al., 1987; Lipp and Pott, 1988; Crespo et al., 1990; Niggli and Lederer, 1993). This possibility is strongly supported by the finding that the current is rapidly and reversibly abolished when external Na^+ was substituted for Li^+ (Fig. 4*b*). Specific antagonists of $\text{Na}^+/\text{Ca}^{2+}$ exchangers are not available, however, in vertebrate heart cells and photoreceptors, quinidine has been reported to partially block the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Desilets and Horackova, 1984; Lagnado and McNaughton, 1991). When added to the bath at a concentration of 200 μM , quinidine also consistently reduced the flash-induced inward current in *Drosophila* photoreceptors by ca. 50% in a reversible manner (4 out of 4 cells, data not shown).

Arguably the absence of any other current capable of being activated by Ca^{2+} might reflect the inability of the caged compound to diffuse throughout the cell (e.g., into the microvilli). Although this possibility cannot be entirely ruled out (see Discussion), in several cases the cells were allowed to equilibrate with the pipette solution for over twenty minutes rather than the standard 2 min equilibration time normally used. Even so, no other current could be detected beyond that attributable to the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Furthermore, as detailed below virtually instantaneous effects of the caged Ca^{2+} on light-sensitive channel activity can be directly demonstrated.

Effect of caged Ca^{2+} released in WT photoreceptors

Lack of effect during the latent period. The experiments with “blind mutants” described above allow effects of caged Ca^{2+} to be examined without contamination by light-induced channel activity; however, in principle it could be argued that the light-sensitive channels are either not present in these mutants, or for some reason are incapable of being activated by the native messenger(s). In fact substantial evidence suggests this is not the case (see Discussion); nevertheless, the response to caged Ca^{2+} was also examined in wild type (WT) photoreceptors.

The rapid activation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger described above indicates that caged Ca^{2+} is being released, as expected, on a submillisecond time scale. Since the light response has a finite latency (>5 msec), if caged Ca^{2+} activates channels either directly, or via a late stage of the transduction cascade, it should be possible to detect this during the first few milliseconds following the photolytic flash. For these experiments, which suffer

Figure 4. Voltage dependence of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger current, and dependence on sodium. *a*, Response to a voltage ramp from -80 mV to $+80$ mV applied before (upper trace) and immediately after a 300V Xe flash (arrow) in an *ora* photoreceptor loaded with 5 mM DM-nitrophen/4 mM Ca^{2+} via a Cs/TEACl electrode. The graph shows the normalized I – V relationship of the current attributable to the release of caged Ca^{2+} obtained from subtracting the two traces above plus one other I – V curve obtained from another photoreceptor in the same manner. Data have been fitted with an exponential function of the form $I = I_0 \cdot \exp(zFE/2RT)$, where $z = 0.76$ (corresponding to an e -fold change per 76 mV). *b*, Two responses from the same *ora* photoreceptor bathed first in normal Ringer's (Na^+) and then ca. 10 sec after washing to an identical Ringer's with Li^+ substituted for Na^+ . Both recordings at -50 mV. The inward current is completely abolished in the absence of external Na^+ .

from the inevitable drawback that sensitivity to light never recovers following the photolytic flash, responses were recorded in 100 μM Ca^{2+} Ringer's to slow the kinetics of the light-induced current itself (Fig. 5). At negative holding potentials (-20 mV, 10 cells; -50 mV, 5 cells), the only response recorded during the first 5 msec in cells loaded with Ca/DM-nitrophen is the biphasic ERC superimposed upon a ca. 20–40 pA inward current. Unlike the light-induced current this inward current does not reverse ($+50$ mV, six cells; $+80$ mV, three cells), is absent

Figure 5. Effects of caged Ca^{2+} in WT photoreceptors. Wild type adult photoreceptors recorded with electrodes containing CsCl/TEACl and Ca/DM-nitrophen, bathed in $100 \mu\text{M}$ Ca^{2+} Ringer's. At a holding potential of -20 mV (*a*) the Xe flash (arrow) evokes a large light-induced inward current. Examination of the first 5–10 msec of the response at higher gain (*right*) reveals a biphasic early receptor current superimposed upon a small inward current similar in size to that previously attributed to the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (ca. 20 pA; see dotted baseline and arrow-head) and absent in the control (loaded with 5 mM EDTA : 3 mM Ca^{2+}). *b*, At a holding potential of $+50$ mV, the LIC is now outward, but the putative exchanger current is either not detectable, or still inward (compare control).

in controls (14 cells) and presumably again represents the electrogenic $\text{Na}^+/\text{Ca}^{2+}$ exchanger. The latency of the light-induced current following photolysis of Ca/DM-nitrophen (6.9 ± 1.0 msec, $n = 10$ at -20 mV) was not significantly different from that recorded in controls loaded with Ca/EDTA (6.8 ± 1.8 msec, $n = 5$). There was some indication that the maximum responses elicited with Ca/DM-nitrophen (5.9 ± 1.7 nA, after applying a series resistance correction, at -20 mV), were larger than controls (5.2 ± 1.7 nA); however, because of the poor voltage-clamp control with these large currents this latter point could not be accurately substantiated.

Effects of caged Ca^{2+} released during the light response. A number of studies have indicated that the light-induced current can be modulated by Ca^{2+} influx (Hardie, 1991a; Ranganathan et al., 1991; Hardie and Minke 1994b). The question arises whether these modulatory effects can be mimicked by caged Ca^{2+} ; if so this would provide further support for the modulatory roles of Ca^{2+} , and also serve as a positive control that the caged Ca^{2+} has access to the light-sensitive channels. In order to test the effects of caged Ca^{2+} on light-dependent channel activity,

Figure 6. Facilitation of the light-induced current (LIC) by Ca/DM-nitrophen. *a*, WT adult photoreceptors clamped at -20 mV with electrodes containing Cs/TEACl bathed in Ca^{2+} free Ringer's. During the time indicated by the stimulus marker, a step of 360 nm light is delivered from a 75 W Xe lamp. This is sufficient to saturate the LIC, but releases little caged Ca^{2+} (Fig. 1). At the time indicated by the arrows the Xe flash is discharged. In the controls (*upper two traces*) recorded with electrodes containing 5 mM EDTA : 3 mM Ca^{2+} (*top trace*) or unloaded DM-nitrophen (*second trace*), the flash induces an ERC, but no detectable deflection in the response waveform. With Ca/DM-nitrophen (*lower trace*), release of caged Ca^{2+} evokes a pronounced and immediate increase in the inward current. *b*, Responses recorded as above: each trace is from a different WT photoreceptor recorded from the same preparation (adult photoreceptors, bathed in $100 \mu\text{M}$ Ca^{2+} Ringer's), but clamped at different holding potentials. The current activated by the photolytically released Ca^{2+} reverses at approximately the same voltage as the LIC itself, that is, ca. 15 mV.

cells were first stimulated with a saturating step of light (360 nm, 200 msec), which by itself is not of sufficient intensity to release significant Ca^{2+} from the cage (see Fig. 1). Subsequently, the Xe flash lamp was discharged at various times following the onset of the test flash. Numerous controls (using Ca/EDTA, unloaded DM-nitrophen or Mg/DM-nitrophen instead of Ca/DM-nitrophen in the electrode) under a variety of conditions show that with this paradigm the Xe flash by itself produces no obvious response from the cell apart from the ERC (e.g., Figs. 6, 7).

By contrast, when the cells have been loaded with Ca/DM-nitrophen, the photolytic flash elicits clear responses in every cell tested (over 70 cells). The nature of the response depends upon the time of presentation. When the Xe flash is delivered during the rising phase of the response (Fig. 6), there is a rapid and pronounced facilitation of the light response with submillisecond latency. Ca^{2+} influx via the light-sensitive channels itself generates a facilitation of the light response which is enhanced at hyperpolarized holding potentials (Hardie, 1991a; Hardie and Minke, 1992). To reduce this source of facilitation, these experiments were performed in Ca^{2+} free or low Ca^{2+} (100 μM) Ringer's and relatively depolarized voltages (typically, -20 mV). It was impractical to accurately quantify this signal, as these large currents are poorly clamped and because it was impossible to obtain more than one response from a single cell with these bright lights; however, it is clear that the magnitude of the facilitated current (several nA in some cases) greatly exceeds the signal which was previously attributed to the $\text{Na}^+/\text{Ca}^{2+}$ exchanger current. Furthermore, unlike the exchanger current this signal reverses, and large (up to 1 nA or more) outward currents can now be observed at depolarized potentials (see below). Similar virtually instantaneous facilitation by caged Ca^{2+} can in fact also be clearly observed during much smaller, non-saturating responses although now the Xe flash obviously also induces an additional later light response (data not shown).

Recent evidence suggests that the light-induced current in *Drosophila* is mediated via at least two different conductances with different reversal potentials (E_{rev}): a Ca^{2+} permeable channel (*trp* dependent) with a relatively depolarized E_{rev} , and a nonselective cation channel with E_{rev} around 0 mV, which is responsible for the residual response in the *trp* mutant (Hardie and Minke, 1992). It was therefore of interest to measure E_{rev} of the current activated by the release of caged Ca^{2+} during the light response. These experiments cannot be performed in the same cell, but traces from cells recorded sequentially in the same preparation and clamped at different holding potentials are superimposed in Figure 6b. The signal reverses between $+10$ and $+15$ mV, that is, at approximately the same potential as the light-induced current itself, and considerably more positive than E_{rev} for the non-*trp*-dependent channels (see further below).

Ca^{2+} -dependent inactivation

If the photolytic flashes are presented at later stages of the response a completely different behavior is observed (Fig. 7). Namely the LIC is now rapidly and almost completely suppressed, again with almost negligible latency. It is difficult to precisely pinpoint at which phase of the response facilitation gives way to inactivation. In some cases an immediate inhibitory effect of the photolysis can be seen even after only 125 msec, during the falling phase of the peak response and before the response has reached a stable plateau level. A pronounced and rapid inactivation is always seen once the plateau level has been reached (typically ca. 150 msec after the onset of the light response). The time constant of inactivation, taken from the single exponential functions fitted to the decay elicited during the plateau phase is rather rapid (3.5 ± 1.0 msec, $n = 9$ at -50 mV in normal, 1.5 mM Ca^{2+} Ringer's).

A characteristic of whole-cell recordings from dissociated *Drosophila* photoreceptors is that after a certain period of time, there is a spontaneous activation of cation selective channels in the plasma membrane (Hardie and Minke, 1994a,b). The properties of this "rundown current" or RDC strongly suggest that

Figure 7. Inactivation of the LIC by DM-nitrophen in WT photoreceptors. *a*, A saturating 360 nm stimulus induces a distinct peak and plateau (the peaks are clipped due to saturation of the amplifier). In *a*, recorded with Ca/DM-nitrophen a Xe flash delivered during the plateau induces a rapid inactivation (single exponential decay, time constant ca. 3 msec). On a faster time scale the response can be seen to inactivate from a level negative to the preceding plateau level. It is unclear whether this simply represents the contribution of the ERC and inward $\text{Na}^+/\text{Ca}^{2+}$ exchange current or whether there is also a rapid facilitation before the onset of the inactivation. *b*, Control using electrode containing Ca/EDTA. The Xe flash now induces a clear ERG but otherwise has no effect. Responses recorded at -50 mV in adult photoreceptors with K-gluconate electrodes in normal (1.5 mM Ca^{2+}) Ringer's.

it is mediated by the light-sensitive channels themselves, uncoupled from the phototransduction machinery. It was interesting therefore to ask whether the photolysis of caged Ca^{2+} produces similar effects on this spontaneous channel activity. These experiments can be relatively easily performed since following development of the rundown current the photoreceptors were incapable of responding to more than one saturating flash, and hence the effect of the caged compounds on channel activity could be investigated without contaminating effects of the light itself. Large rundown currents also develop readily in the *ora* mutant (Hardie and Minke 1994a) allowing the effects of caged Ca^{2+} to be studied without contamination by the ERC. Figure 8, recorded in *ora* (and typical of responses in all of over 30 cells in both WT and *ora*) shows that the effects of caged Ca^{2+} on the RDC are very similar to those on the LIC itself. At

Figure 8. Caged Ca^{2+} facilitates and inactivates the rundown current. Traces recorded after the development of spontaneous rundown current (RDC) in an *ora* photoreceptor loaded with $\text{Ca}/\text{DM-nitrophen}$ in a Cs/TEACl electrode. A voltage step to +80 mV (from a holding potential of -50 mV) evokes a slowly developing outward current attributable to the outwardly rectifying RDC. The Xe flash elicits an initial outward current (facilitation) followed by a pronounced inhibition. At -50 mV the Xe flash induces a similar, inverted response, but much smaller as the RDC is already strongly inactivated by hyperpolarization (Hardie and Minke, 1994b). The initial inward current at -50 mV is probably a combination of $\text{Na}^+/\text{Ca}^{2+}$ exchanger current and facilitation of the RDC. *b*, Response at -50 mV from another *ora* photoreceptor on an expanded scale. The RDC is associated with appreciable high frequency channel noise (lower control trace). Following the Xe discharge the rapid inactivation clearly also suppresses the noise in the RDC.

hyperpolarized potentials the major effect appears to be Ca^{2+} -dependent inactivation of the chronically activated channels. There may in addition be some early facilitation (i.e., inward current) but this cannot be reliably distinguished from the $\text{Na}^+/\text{Ca}^{2+}$ exchange current which is also inward. At depolarized potentials an early facilitation is clearly distinguishable as the currents are now outward and also strongly outwardly rectifying; however, again the dominant effect is a rapid inactivation of the channels. At hyperpolarized potentials in particular it is apparent that the channel noise which is characteristic of the RDC (Hardie and Minke, 1994a) is also largely abolished following the photolytic flash (Fig. 8*b*). The rate of inactivation of the RDC by caged Ca^{2+} is comparable to that of the light-induced current ($\tau = 2.3 \pm 0.8$ msec, $n = 6$ at -50 mV).

trp

The above experiments indicate that the photolytically released Ca^{2+} has very rapid access to the light-sensitive channels (or to another target which itself can control the light sensitive channel activity with a submillisecond latency). However, since there are presumed to be at least two classes of light-sensitive channel (Hardie and Minke, 1992) it could be argued that only one of these is accessible to the caged Ca^{2+} . The effects of caged Ca^{2+} on the light response were therefore also investigated in the *trp* mutant which is believed to lack one of the two suspected classes of channel.

As in wild type photoreceptors, the effect of photolysis of caged Ca^{2+} appears to depend upon the timing of the photolytic flash, but the behavior shows marked differences. If the Xe flash is discharged during the early phase of the response, there is no indication of a stimulus coincident effect of the released Ca^{2+} beyond effects attributable to the ERC or the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. However, compared to control responses elicited without DM-nitrophen in the electrode, a significant overall inhibition of the light response is apparent (Fig. 9). Peak responses are ca. 50% of control values, and the decay to baseline is greatly accelerated (time to decay to 50% peak response following Xe flash: 26 ± 14 msec, $n = 6$ in cells loaded with $\text{Ca}/\text{DM-nitrophen}$; cf. 105 ± 16 msec, $n = 5$ with Ca/EDTA controls). A similar comparison holds for responses elicited by the Xe flash alone (Fig. 9*a*). When loaded with $\text{Ca}/\text{DM-nitrophen}$ responses are smaller and decay rapidly, while without DM-nitrophen a larger more slowly decaying response is elicited. As in wild type, these experiments with the Xe flash alone also show that there is no detectable effect of the caged Ca^{2+} in the latent period, beyond activation of a small current attributable to $\text{Na}^+/\text{Ca}^{2+}$ exchange (Fig. 9*a*, inset).

In marked contrast, if the Xe flash is discharged during the falling phase of the response to the test flash, an inward current with no detectable latency is clearly detected at negative holding potentials. The magnitude of this is somewhat variable, but consistently larger than the current previously attributed to the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (e.g., Fig. 9*c*). Furthermore, as the cell is depolarized this response readily reverses with a reversal potential around 0 mV (Fig. 10*a*). This is similar to E_{rev} of the *trp* light-induced current, but clearly more negative than that of the equivalent responses recorded in WT (cf. Fig. 6*b*). In addition to this facilitatory effect, in some cells a delayed inhibitory effect of the caged Ca^{2+} was also apparent during these late phases. However, this was much less marked and less rapid than the equivalent Ca^{2+} -dependent inactivation characteristic of responses in WT.

The activation of a current reversing at ca. 0 mV during the falling phase of the response indicates that at this stage of the response Ca^{2+} is also capable of activating or facilitating the light-sensitive channels in *trp*. It is interesting to ask whether the potency of Ca^{2+} in this respect is strictly limited to the time when the channels are open, or if there is a finite time after the decay of the LIC when Ca^{2+} is still capable of activating the light-sensitive channels. For example it is possible that the channels have a dual requirement for Ca^{2+} and a second diffusible messenger which might then be expected to persist after the response has decayed. Figure 10*b* shows a series of responses in different cells recorded sequentially in the same preparation, where the Xe flash is discharged at different stages during the decay of the response. In order to distinguish any facilitation

Figure 9. Effects of Ca/DM-nitrophen in *trp*. *a*, Responses to Xe flashes alone in *trp* photoreceptors loaded with Ca/DM-nitrophen or with Ca/EDTA (control) electrodes. With caged Ca²⁺ release the overall response is much reduced and decays more rapidly. The inset reveals that there is also an early small inward current of ca. 80 pA attributable to the Na⁺-Ca²⁺ exchanger while in the control (lower trace) only the ERC is visible (in the upper trace an unusually large flash artefact has obscured the ERC). *b*, Xe flash discharged during the rising phase of the response to a saturating 200 msec 360 nm pulse. Again compared to the control release of caged Ca²⁺ causes a significant suppression and more rapid decay of the response. On an expanded scale however, no immediate effect of the caged Ca²⁺ can be detected. *c*, Photolysis of Ca/DM-nitrophen during the falling phase of the response induces a rapid facilitation of the inward current. In the control only the ERC can be seen (insets on expanded time base). *d*, In WT photoreceptors treated with La³⁺ (20 μM in bath) caged Ca²⁺ released during the falling phase induces a significant facilitation. All traces recorded at -50 mV in adult photoreceptors.

unequivocally from the inward Na⁺/Ca²⁺ exchange current, the experiments were performed at +40 mV thus generating outward currents. It appears that the potency of Ca²⁺ is indeed rather strictly limited to the period when the channels are open and even 25 msec after the response has decayed (middle trace) no further channels are activated by the caged Ca²⁺—although a small inward current attributable to the Na⁺/Ca²⁺ exchanger can still be seen.

trp-like effects in WT

All aspects of the *trp* phenotype tested have been shown to be mimicked by micromolar concentrations of the Ca²⁺ channel blocker, La³⁺ (Hochstrate, 1989; Suss-Toby et al., 1991; Hardie and Minke, 1992, 1994a). It is therefore not surprising to find that the effects of caged Ca²⁺ in WT photoreceptors bathed in 20 μM La³⁺ are similar to those in *trp*. In particular, Figure 9*d* shows that a photolytic flash delivered during the falling phase of the response now induces clear facilitation but no rapid inactivation.

Effects of nitr-5

Nitr-5 is an alternative caged Ca²⁺ compound belonging to the BAPTA class of chelators (Adams et al., 1988). Like BAPTA, nitr-5 has very rapid buffering kinetics, no significant affinity for Mg²⁺ and little sensitivity to pH. However, the change in affinity for Ca²⁺ on photolysis is much less than is the case for DM-nitrophen so that smaller Ca²⁺ increments result (ca. 1–2 μM cf. ca. 20 μM for DM nitrophen; see Materials and Methods). The effects of nitr-5 were tested to explore the effects of a lower range of Ca²⁺ concentrations; furthermore the absence of significant Mg²⁺ and pH effects served as a useful further control that the effects of DM-nitrophen can be attributed to Ca²⁺.

Despite the much lower Ca²⁺ concentrations achieved, some of the effects observed with DM-nitrophen could also be elicited

by flash photolysis with nitr-5; however, others could either not be detected or were much reduced (Figs. 1, 11). Specifically: (1) the inward current attributed to the Na⁺/Ca²⁺ exchanger (in *ora*, *norpA*, or inactivated *trp*) could be detected under favorable recording conditions, but was much reduced in magnitude, averaging 4 pA ± 2 at -50 mV, that is, less than 10% of the size seen with DM nitrophen (e.g., Fig. 1). (2) Facilitation during the rising phase of the light response in WT could not be reliably detected. Possibly a minor effect is still present, but it cannot be reliably distinguished from the ERC (data not shown). (3) Strikingly, however nitr-5 was equally effective at inactivating the light-sensitive channels, both late in the response to light and after the development of the rundown current. In particular, the time constants of inactivation induced by photolysis of nitr-5 for both the LIC and the RDC were as rapid as those induced by DM-nitrophen ($\tau = 2.3 \pm 0.6$ msec; Fig. 11). (4) In *trp*, a slight facilitation of the LIC was sometimes elicited during the falling phase of the response (data not shown), but no evidence for suppression or inactivation of the response could be detected.

Discussion

The present study has explored the effects of photolytically released Ca²⁺ in *Drosophila* photoreceptors, using three different strategies to avoid contamination by the light response itself: (1) using visually nonresponsive (“blind”) mutants, (2) exploiting the finite latent period of the light response, (3) saturating the photoreceptor response with a stimulus which itself is insufficient to release significant quantities of Ca²⁺. Experiments on visually responsive cells suffer from the drawback that, at best cells recover only limited sensitivity to light following these bright stimuli, and also because the large currents activated are only poorly clamped. While this compromises quantitative analysis, qualitatively the effects of caged Ca²⁺ under these conditions were extremely reproducible and seen in every cell tested.

Figure 10. *a*, Reversal potential (E_{rev}) of facilitated current in *trp*. Each trace is from a different cell recorded in the same preparation (adult *trp*), but at a different holding potential. The Xe flash induces a facilitation which reverses at ca. 0 mV, that is, at the same potential as the LIC itself in *trp* (cf. Fig. 6*b*). After the decay of the response in all cases there is a residual inward current, which represents the contribution of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger current. This contaminates the signal due to facilitation of the light-sensitive channels, resulting in a slight overestimate of E_{rev} (i.e., E_{rev} of the facilitation is presumably slightly more negative than 0 mV—as is the E_{rev} of the LIC in *trp*). *b*, Three *trp* cells from the same preparation (adult), loaded with Ca/DM-nitrophen and recorded at a holding potential of +40 mV. The Xe flash is discharged with different delays; only during the response can an outward current (facilitation) be observed. After the response has decayed only the ERC and a small inward current ($\text{Na}^+/\text{Ca}^{2+}$ exchanger) can be detected.

The temporal resolution of the technique is limited by the flash artefact (ca. 200 μsec), the time course of the flash (ca. 1 msec to peak), and the time course of photolytic release of Ca^{2+} (reported as ca. 200 μsec for both DM-nitrophen and nitr-5: Kaplan and Ellis-Davies, 1988). In photoreceptors containing rhodopsin, the response is also contaminated by the early receptor current, which has a duration of ca. 2–3 msec. However, this is a predictable signal which can in principle be subtracted from the traces. The onset of the small inward current activated in “blind” mutants, which is attributed to the $\text{Na}^+/\text{Ca}^{2+}$ exchange current is likely to approximate the time course of the actual Ca^{2+} release and can be used as a benchmark to compare the kinetics of other responses elicited by flash photolysis. In practice, the effects of the released Ca^{2+} can be detected within ca. 500 μsec (Fig. 12).

Figure 11. Effects of nitr-5 in adult WT photoreceptors. *a*, In 1.5 mM Ca^{2+} Ringer's a flash discharged during the plateau of the response generates a rapid inactivation of the LIC (time constant 2.5 msec in this case). The initial inward current is at least partially caused by the ERC. Recorded at –50 mV with electrode containing K-gluconate, 5 mM nitr-5: 4 mM Ca^{2+} . *b*, Following development of the rundown current (RDC), photolysis of nitr-5 induces rapid inactivation of the RDC at both +80 mV and –50 mV. Compared to the effects of DM-nitrophen (Fig. 8), the major difference is the absence of any pronounced facilitation at depolarized potentials. Recorded with CsCl/TEACl electrode and 5:4 Ca-nitr-5.

The $\text{Na}^+/\text{Ca}^{2+}$ exchange current

At resting potential, photolysis of DM-nitrophen, generating a rise in Ca^{2+} to ca. 20–50 μM activates a small (up to 100 pA) inward current. The absolute dependence on extracellular Na^+ , partial block by quinidine, lack of any channel noise, inability to reverse the current and exponential I – V relationship indicate that it represents activation of an electrogenic $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Photolysis of nitr-5, generating ca. 1–2 μM Ca^{2+} , is barely sufficient to activate this current. The size of the current elicited with Ca/DM-nitrophen (mean 76 pA) allows a lower estimate to be made of the capacity of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in *Drosophila* photoreceptors (ca. 18 pmol/sec/ cm^2 at resting potential, assuming a cell capacitance of 45 pF and specific membrane capacitance of 1 $\mu\text{F}/\text{cm}^2$). This is rather high compared to values usually reported in neurons but similar to that reported in *Limulus* photoreceptors (16 pmol/sec/ cm^2 : O'Day and Gray-Kel-

ler, 1991). Since the magnitude was unaffected by the substitution of internal K^+ for Cs^+ , apparently the exchanger is not a $Na^+/Ca^{2+}-K^+$ exchanger as reported in vertebrate photoreceptors (rev., McNaughton, 1990). Like the Na^+/Ca^{2+} exchanger in heart (Desilets and Horckova, 1984) and the $Na^+/Ca^{2+}-K^+$ exchanger in vertebrate photoreceptors (Lagnado and McNaughton, 1991), the *Drosophila* photoreceptor Na^+/Ca^{2+} exchanger is partially (ca. 50%) blocked by 200 μM quinidine. However, it is insensitive to 20 μM La^{3+} , a concentration which completely blocks the *trp*-dependent channels (data not shown). Although the existence of an electrogenic Na^+/Ca^{2+} exchanger in insect photoreceptors is generally accepted (Minke and Armon, 1984; Minke and Tsacopolous, 1986), these are the first experiments allowing direct measurements of the Na^+/Ca^{2+} exchange current in insect photoreceptors, and indeed activation of the Na^+/Ca^{2+} exchange current by caged Ca^{2+} has only very recently been reported (in cardiac myocytes, Niggli and Lederer, 1993). As also concluded by these authors, the reliability of the signal should make caged Ca^{2+} a useful tool for further quantitative analysis of the Na^+/Ca^{2+} exchanger and its role in *Drosophila* photoreceptors.

Ca²⁺ and excitation

The primary aim of this study was to test the hypothesis that Ca^{2+} activates gates at least one class of light-sensitive channel. This hypothesis is based on substantial evidence from a variety of arthropod photoreceptors showing that Ca^{2+} is required for excitation and that under certain conditions can lead to excitation or facilitation. The most direct evidence comes from *Limulus* where pressure injection of Ca^{2+} , either by itself or buffered at 5–45 μM with di-bromo-BAPTA is capable of evoking an inward current with properties similar to the light-induced current (Payne et al., 1986; Shin et al., 1993). Furthermore, prolonged exposure to Ca^{2+} -free Ringer's severely diminishes sensitivity in *Limulus* presumably by depleting Ca^{2+} stores (Bolsover and Brown, 1985), and injection of high concentrations of BAPTA or di-bromo-BAPTA inhibits the light response (Frank and Fein, 1991; Shin et al., 1993). Nevertheless, the involvement of Ca^{2+} has been questioned since the rise in intracellular Ca^{2+} is reported to lag behind the electrophysiological response (Stieve and Benner, 1992; Payne, personal communication) and because Ca^{2+} -activated channels have yet to be discovered in excised patches of light-sensitive membrane (Bacigalupo et al., 1991). One resolution of this apparent paradox is provided by evidence suggesting two or three parallel pathways, possibly mediated by different G-proteins (Frank and Fein, 1991; Nagy, 1991; Nagy et al., 1993). However, Shin et al. (1993) found no component of the light response which was resistant to Ca^{2+} buffering and argued that only one pathway was involved, with Ca^{2+} as an obligatory and sufficient intermediate. In *Drosophila*, it has previously been shown that Ca^{2+} influx during the response (induced either by rapidly raising external Ca^{2+} or simply by hyperpolarizing the cell during the response) leads to pronounced facilitation (Hardie, 1991a). An absolute requirement for Ca^{2+} has been suggested by the finding that during a critical developmental stage, *Drosophila* pupal photoreceptors are incapable of responding to light unless cytosolic Ca^{2+} is artificially increased into the micromolar range (Hardie et al., 1993). We suggested that the requirement for Ca^{2+} was actually not in the cytosol per se, but in the $InsP_3$ -sensitive stores and that for some reason (such as reduced se-

b)

	WT LIC	WT RDC	<i>trp</i> LIC
DM-nitrophen (~20 μM Ca^{2+})			
Facilitation	+ early	+	+ late
Inactivation	+ late	+	(+ early)
nitr-5 (~1 μM Ca^{2+})			
Facilitation	-	-	(+ late)
Inactivation	+ late	+	-

Figure 12. Summary of effects of DM-nitrophen and nitr-5. *a*, Comparison of time courses of photolytic flash (*Xe*), Na^+/Ca^{2+} exchange current (recorded in *ora*), facilitation and inactivation of the LIC in WT, all induced by photolysis of Ca/DM -nitrophen, plotted at different gains as indicated. The two dotted lines (the first indicating the trigger for the flash) are separated by 1 msec showing that facilitation in both WT and *trp*, and probably inactivation as well, are already initiated within 1 msec of the *Xe* flash. *b*, Table summarizing the effects of photolysis of DM-nitrophen (generating ca. 20 μM Ca^{2+}) and nitr-5 (generating ca. 1 μM Ca^{2+}) on the light induced current (LIC) in WT and *trp* and also the rundown current (RDC) in WT. Weak effects in brackets: early/late indicates whether the effect is produced during the rising (early) or falling/plateau (late) phases of the response.

questering ability) at this early developmental stage the stores were effectively empty and could only sequester sufficient Ca^{2+} if cytosolic levels were artificially raised.

The most obvious hypothesis emerging from this body of evidence would be that Ca^{2+} released from the intracellular stores (by $InsP_3$) gates directly (or via an intermediate) at least one class of light-dependent channel (Payne and Fein, 1986; Nagy, 1991; Hardie and Minke, 1993; Shin et al., 1993). In the present study however, the inability of caged Ca^{2+} to activate any conductances beyond a current attributable to the Na^+/Ca^{2+} exchanger suggests that Ca^{2+} may not be sufficient for excitation in *Drosophila*. Before accepting this negative result, however, a number of issues need to be discussed.

The failure of caged Ca^{2+} to activate the light-sensitive channels can be most clearly demonstrated in mutants which fail to respond to light, begging the question of whether the mutants used have light-sensitive channels capable of being activated in the first place. Because of this potential objection, the experi-

ments were performed on three completely independent mutants: *ora* (lacking rhodopsin), *norpA* (lacking PLC), and inactivated *trp*. Of these both *ora* and *norpA* are believed to have the normal complement of light-sensitive channels, while *trp* lacks one class of channel. The presence of functionally intact light-dependent channels in *norpA* is suggested by temperature-sensitive alleles of this mutation which respond normally at permissive temperatures (Pak, 1991; Minke and Selinger, 1992). For the case of *ora* the competence of the transduction cascade could also be directly demonstrated in the allele used in these experiments, since some cells still generated responses, including large quantum bumps, to very bright light. Furthermore the "rundown current," which represents the spontaneous activation of a major component of the light-sensitive conductance, is as large in *ora* as it is in WT indicating that the full complement of light-sensitive channels is present and capable of being activated in this mutant (Fig. 8; Hardie and Minke, 1994a; B. Minke, unpublished results). Immunostaining has also shown that the *trp* gene product (a putative light-dependent channel protein) is normally expressed in *ora* with the same subcellular localization at the base of the microvilli (J. A. Pollock, unpublished observations). Finally, the failure of caged Ca^{2+} to activate an inward conductance, beyond that attributable to $\text{Na}^+/\text{Ca}^{2+}$ exchange could also be directly demonstrated in WT photoreceptors during the time allowed by the finite latent period of the response to the Xe flash (ca. 5 msec).

A second potential objection is that the concentrations achieved by the caged Ca^{2+} released in these experiments (maximally estimated at 20–50 μM) are insufficient to gate the channels. Additionally, and of potentially greater concern, it is possible that at least a subpopulation of the light-sensitive channels are diffusionally inaccessible, and indeed there are indications that substances applied via the patch pipette have difficulty entering the microvilli in *Drosophila* (R. Hardie and B. Minke, unpublished observations; C. Zuker personal communication). Arguing against both these possibilities are the effects of flash photolysis during the light response, where it was shown that both *trp*-dependent and *trp*-independent channels are affected by the Ca^{2+} with a latency of less than 1 msec. Similar effects were seen on the "rundown current," believed to represent spontaneously activated channels uncoupled from the transduction cascade (Hardie and Minke, 1994a).

The possibility remains that caged Ca^{2+} does not have access to the channels, and that the effects of caged Ca^{2+} on activated channels are indirectly mediated. Although this possibility cannot be excluded, if the hypothesis that Ca^{2+} is the final messenger is still to be entertained on this basis it would imply an unusual degree of compartmentalization and/or extremely localized and rapid control of Ca^{2+} . The difficulty of such an interpretation is highlighted by considering the most obvious putative upstream target for regulation of the light-activated conductance by caged Ca^{2+} , namely, the InsP_3 receptor (Ca^{2+} release channel), which is reported to be both facilitated and inhibited by Ca^{2+} (Baumann and Walz, 1989; Payne et al., 1990; Bezprozvanny et al., 1991; Finch et al., 1991). The hypothesis that Ca^{2+} is a messenger of excitation assumes that the source of the Ca^{2+} is from the InsP_3 receptor itself. Self-evidently, on this hypothesis, Ca^{2+} released from the stores must have access to the channels (or an intermediate which itself can activate the channels). It is then difficult to envisage how the photolytically released Ca^{2+} could regulate the InsP_3 receptor without having the same access to light-sensitive channels.

Ca²⁺-dependent facilitation and inactivation

Facilitation can be seen in both WT and *trp* photoreceptors, although in *trp* the facilitation can only be clearly distinguished when the Ca^{2+} is released during the falling phase of the response, while in WT it is particularly evident when applied during the rising phase. In WT photoreceptors the time course and magnitude of the facilitation are very reminiscent of the facilitation of the light response observed on applying hyperpolarizing voltage steps during the light response (Hardie, 1991a; Hardie and Minke, 1994b). Previously this was attributed to enhanced Ca^{2+} influx via the *trp*-dependent channels (Hardie and Minke, 1992); the present results with caged Ca^{2+} add strong support to this interpretation. The question arises whether it is the *trp*-dependent or the *trp*-independent channels which are facilitated—or both. The two putative channels can be distinguished by their reversal potentials (ca. +15 mV and 0 mV, respectively; Hardie and Minke, 1992); in both WT and *trp* the reversal potential of the facilitation appears to coincide with the respective reversal potential of the LIC suggesting that both channels are subject to this facilitation. The very rapid time course of the facilitation with a latency of less than 1 msec suggests that the Ca^{2+} may be acting at the level of the channels themselves. This possibility is supported by the observation of facilitation of the so-called rundown current (RDC), the properties of which—in particular the associated high frequency "channel noise"—suggest that it represents spontaneous activity of light-sensitive channels uncoupled from the transduction cascade (Hardie and Minke, 1994a).

A particularly dramatic effect of caged Ca^{2+} release in these experiments is the rapid inactivation of the light-sensitive conductance. Once again, since the RDC is also subject to inactivation, and because the time course is so fast (2–3 msec time constant), it seems possible that the site of action is the channels themselves. This is consistent with recent independent evidence indicating that the light-dependent channels are directly inactivated by Ca^{2+} influx under some conditions (Hardie and Minke, 1994b). Surprisingly the Ca^{2+} -dependent inactivation of the light sensitive channels appears already to be saturated by ca. 1 μM Ca^{2+} , since the inactivation—whether during the light response or during rundown—was as pronounced and fast with nitr-5 as it was with DM-nitrophen generating levels of 20 μM or higher. By contrast the facilitation seen during the rising phase of the response was much more pronounced with the higher Ca^{2+} step generated by DM-nitrophen. This at first seems counterintuitive since it implies that during a continuous rise in Ca^{2+} the LIC should be inactivated before being facilitated—the opposite of what is observed (Hardie, 1991a). The possibility needs to be considered therefore that the channels (or preceding step) actually develop a susceptibility to Ca^{2+} -dependent inactivation during the response.

Conclusion

The most significant finding of these studies is a negative one, namely, the inability to directly activate the light-dependent channels by release of caged Ca^{2+} , although the Ca^{2+} apparently has access to both *trp*-dependent and *trp*-independent channels, or a site which itself can regulate their activity with a submillisecond latency. These results do not, therefore support the hypothesis that a rise in cytosolic Ca^{2+} is a sufficient messenger of excitation in *Drosophila*; they do, however, support a powerful facilitatory role for Ca^{2+} in phototransduction.

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