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INDO-1 Measurements of Absolute Resting and Light-Induced Ca^{2+} Concentration in *Drosophila* Photoreceptors

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Absolute Ca^{2+} levels in dissociated *Drosophila* photoreceptors were measured using the ratiometric indicator dye INDO-1 loaded via patch pipettes, which simultaneously recorded whole-cell currents. In wild-type photoreceptors, the ultraviolet (UV) excitation light used to measure fluorescence elicited a massive Ca^{2+} influx that saturated the dye (>10 μM Ca^{2+}), but lagged the electrical response by 2.8 msec. Resting Ca^{2+} levels in the dark, measured during the latent period before the response, averaged 160 nM in normal Ringer's (1.5 mM Ca^{2+}). Ca^{2+} increases in response to weak illumination were estimated (1) by using a weak adapting stimulus before the UV excitation light and measuring Ca^{2+} during the latent period; and (2) by using *ninaE* mutants with greatly reduced rhodopsin levels. Ca^{2+} rose linearly as a function of the time integral of the light-sensitive current with a slope of 2.7 nM/pC. In the transient receptor potential (*trp*) mutant, which lacks a putative light-sensitive channel subunit, the slope was only 1.1 nM/pC, indicating a 2.5-fold reduction in the fractional Ca^{2+} current. From these data, it can also be estimated that >99% of the Ca^{2+} influx is effectively buffered by the cell. In Ca^{2+}-free Ringer's, resting cytosolic Ca^{2+} was reduced (to 30–70 nM), but contrary to previous reports, significant light-induced increases (~250 nM) could be elicited. This rise was reduced to <20 nM when extracellular Na^+ was replaced with *N*-methyl-D-glucamine, suggesting that it could be attributed to Na^+ influx altering the Na/Ca exchanger equilibrium. It is concluded that any light-induced release from internal stores amounts to <20 nM.

Key words: calcium entry; inositol phosphates; phototransduction; calcium fluorimetry; INDO-1; Drosophila; *photoreceptor; vision*

Phototransduction in invertebrate photoreceptors is believed to be mediated by the phosphoinositide (PI) signaling cascade (for review, see Payne et al., 1988; Minke and Selinger, 1991; Nagy, 1991; Hardie and Minke, 1993, 1995; Ranganathan et al., 1995), a ubiquitous G-protein-coupled signaling system characterized by the production of the second messengers inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (for review, see Berridge, 1993). Some of the most compelling evidence for this conclusion comes from analysis of *Drosophila* phototransduction mutants (for review, see Hardie and Minke, 1995; Ranganathan et al., 1995). For example, null mutants of the no-receptor potential A (*norpA*) gene, which encodes a light-activatable phospholipase C (PLC) (Bloomquist et al., 1988; Toyoshima et al., 1990; McKay et al., 1995), are completely unresponsive to light (Pak et al., 1969; Minke and Selinger, 1992), whereas a near null mutation in the PLC-specific G-protein (G_q) α subunit also almost completely abolishes the response to light (Scott et al., 1995).

The ability to genetically manipulate the PI cascade has been complemented by a number of experimental assays, including the ability to measure the light-activated current under whole-cell voltage clamp using a preparation of dissociated ommatidia (Hardie, 1991; Ranganathan et al., 1991). More recently, the same preparation has been used for making simultaneous measurements of intracellular Ca^{2+} using fluorescent Ca^{2+} indicators. To date, only two studies have investigated the light-induced changes in cytosolic Ca^{2+} in *Drosophila* (Peretz et al., 1994a; Ranganathan et al., 1994). Both reported large light-induced Ca^{2+} influx signals, but failed to detect Ca^{2+} rises that might have been attributed to Ca^{2+} release in Ca^{2+}-free solutions. Because both studies used uncalibrated single-wavelength dyes, no information is currently available on absolute Ca^{2+} concentration. In addition, temporal resolution was limited so that measurements, e.g., of latency, were not possible. Both studies recognized an obvious difficulty with Ca^{2+} fluorimetry in photoreceptors; namely, that the measuring light itself induces saturating light responses, often resulting in irreversible damage. An elegant solution to this problem was provided by Ranganathan et al. (1994) by using long-wavelength dyes combined with an ultraviolet (UV)-absorbing rhodopsin substituted transgenically into the photoreceptors. Nevertheless, the measuring light still induced sizeable responses, so that behavior near threshold was not measured. In the present study, cytosolic Ca^{2+} was measured using the ratiometric indicator INDO-1. The data provide the first information on absolute Ca^{2+} levels in *Drosophila* photoreceptors, both in the dark and over a range of adapting intensities. They also demonstrate that there can be substantial light-induced Ca^{2+} increases in the absence of extracellular Ca^{2+}; however, these are most likely attributable to the effects of Na^+ influx on the Na/Ca exchange equilibrium rather than release from intracellular stores. The data also allow an estimation of the effective buffering capacity of the cells and a quantitative comparison of the fractional Ca^{2+} current carried by the light-sensitive channels in wild type (WT) and the transient receptor potential (*trp*) mutant, which has been proposed to lack a subunit of the light-sensitive channels responsible for high Ca^{2+} permeability (Hardie and Minke, 1992).

MATERIALS AND METHODS

Animals. The wild-type strain was white-eyed (*w*) Oregon *Drosophila melanogaster*; in addition, the following mutants were used (on a *w*

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background): trp^{301}, a null mutant of the *trp* gene that encodes a putative light-sensitive channel subunit (Montell and Rubin, 1989; Hardie and Minke, 1992; Phillips et al., 1992; Pollock et al., 1995); and two strains lacking the *ninaE* gene, which encodes the rhodopsin of the principal photoreceptor class R1–R6 (O'Tousa et al., 1985; Zuker et al., 1985). Although both of these strains [$ninaE^{ora}e^{s}$ (referred to as *ora*, kindly supplied by W. Pak, Lafayette, IN) and *ry* $ninaE^{117}e^{s}$ (subsequently referred to as *ninaE*, kindly supplied by J. O'Tousa, Notre Dame, IN)] are reported to be null mutants, R1–R6 photoreceptors in both stocks often had small amounts of functional rhodopsin, possibly attributable to low-level expression of other opsin genes. These mutants were also found to be useful for measurements of light-induced Ca^{2+} influx at low effective intensities. Adult WT and *trp* flies were used up to 4 hr posteclosion; because of age-dependent degeneration, *ora* and *ninaE* flies were taken as late stage (>90 hr) pupae. Flies were reared at 25°C in the dark.

Solutions. The electrode solution contained (in mM): 140 Kgluconate, 4 MgATP, 0.4 Na_2GTP, 2 $MgCl_2$, and 100 μM of either INDO-1, Mag-INDO-1, or Fluo-3. (tetrasodium salts; Molecular Probes, Eugene, OR). The bath contained (in mM): 120 NaCl, 5 KCl, 4 $MgSO_4$, 10 *N*-Tris-(hydroxymethyl)-methyl-2-amino-ethanesulphonic acid, 25 proline, 5 alanine, and either 1.5 mM $CaCl_2$ (normal Ringer's) or no added Ca^{2+} and 1 or 2 mM EGTA (Ca^{2+}-free Ringer's). For some experiments (see Fig. 10), all permeant ions (Na, K, Ca, and Mg) were replaced with 150 mM *N*-methyl-D-glucamine (NMDG) chloride. All solutions were buffered at pH 7.15; experiments were performed at room temperature (20° ± 1°C).

Electrophysiology and Ca^{2+} measurement. Whole-cell recordings from photoreceptors were made from dissociated *Drosophila* ommatidia, as described previously (Hardie, 1991, 1995). Although each ommatidium contains eight photoreceptors, there is no indication of electrical or dye coupling between neighboring photoreceptors; all recordings were made from the single R1–R6 photoreceptors, identified by the whole-cell capacitance, which is at least twice as large as for two minor classes of photoreceptors, R7 and R8.

For ratiometric Ca^{2+} measurements, the dual emission dyes INDO-1 and Mag-INDO-1 were chosen because they allow high sampling rates without the requirement for mechanical chopping of the excitation beam (as with Fura-2). It was recently reported that Ca^{2+} measurements using INDO-1 in vertebrate photoreceptors were contaminated by a change in the binding properties of the dye induced by the measuring light (Gray-Keller and Detwiler, 1994). This problem has not been reported in any other preparation and, in the present experiments, the absence of all but a tiny change in fluorescence in Ca^{2+}-free solutions strongly suggests that there is no significant Ca^{2+}-independent light-induced change in fluorescent properties of the dye. Fluorescence of both INDO-1 and Mag-INDO-1 was excited using 360 nm light delivered via the fluorescent port of a Nikon Diaphot inverted microscope using a DM380 (Nikon, UK) dichroic mirror. For Fluo-3, light at 480 nm was delivered via a DM510 dichroic mirror. Illumination was from a 75 W Xe lamp via a monochromator (Photon Technology Instruments, Brunswick, NJ) and a Uniblitz shutter (Vincent Associates, Rochester, NY) with rise time 1.8 msec. Intensity was controlled by apertures placed at the exit of the monochromator. Fluorescence was collected via a rectangular diaphragm that just covered the recorded cell but excluded the microelectrode, and was measured simultaneously at 405 and 480 nm (INDO-1, Mag-INDO-1) or at wavelengths >520 nm (Fluo-3) via photomultipliers in photon-counting mode.

Background correction. Because of the relatively high UV autofluorescence of the photoreceptors (typically ~30% of total signal), accurate background correction was critical for these measurements. When using *ora* or *ninaE* photoreceptors, the background fluorescence was determined routinely for every cell after giga-seal formation, but before establishing the whole-cell configuration. However, this procedure was considered impractical for WT and *trp* photoreceptors, because the measuring light would have resulted in severe light adaptation before the experiment started. Consequently, background was determined separately on at least two ommatidia at the beginning and end of each preparation. The measuring light induced a small change in the autofluorescence of the photoreceptors over the first 200 msec of illumination, probably because of photoconversion of visual pigment and/or changes in mitochondrial pigments. This change (an ~6% reduction in fluorescence measured at 480 nm) would have manifested itself as a slight (~20–40 nM) increase in Ca^{2+} and was corrected for in measurements of light-induced Ca^{2+} changes in Ca^{2+}-free solutions by subtracting an averaged background template (appropriately scaled) from the raw fluorescence traces before determining the fluorescence ratio. As additional controls, background fluorescence was measured from ommatidia after forming a seal with a dye-filled electrode and also from cells during whole-cell voltage clamp using otherwise identical electrode solutions, but without indicator dyes. Background fluorescence values under these conditions differed by <5% from values determined from intact ommatidia in the absence of electrodes. Background values varied little, apart from a slow reduction over the lifetime of the lamp: from the SD of the background fluorescence (~4% of mean), it was estimated that an error of less than ± 20 nM (SD) was introduced by this indirect method of background correction.

Calibration. Free [Ca_i] was calculated from the ratio (R) of fluorescence at 405 and 480 nm, using the equation:

$$[Ca] = K^d \times (R - R_{min})/(R_{max} - R) \quad (1)$$

(Grynkiewicz et al., 1985). Values for R_{max}, R_{min}, and K^d were all determined *in situ* both in *ninaE* photoreceptors and also using a cell line (*Drosophila* S2 cells). Cells were loaded, as for the experiments, via the patch pipette using solutions similar to the normal electrode solution but containing either no Ca^{2+} (20 EGTA) or 10 mM Ca^{2+} for R_{min} and R_{max}, respectively. To obtain estimates of the effective K^d, three different EGTA–Ca solutions were used with calculated free Ca of 111 nM (10 EGTA:3.5 Ca), 207 nM (10 EGTA:5 Ca), and 483 nM (10 EGTA:7 Ca). At least 5 min was allowed for equilibration before measuring the fluorescence ratio. The ratios obtained for all five solutions (R_{max}, R_{min}, and three different R values) were then fitted to Equation 1 with K^d as free parameter to obtain a value for K^d (1.16 μM). Calibrations were repeated at regular intervals and not found to change significantly over the period of the experiments. Note, however, that small differences in R_{min} can have a large effect on the estimated Ca^{2+} at the lower end of the measured ranges (<100 nM).

Values for Mag-INDO-1 were obtained in an analogous manner except that it was not necessary to obtain an R_{min} value, as resting cytosolic Ca^{2+} is negligible for this low-affinity dye so that R_{min} could be taken from the resting value in any given cell, and K^d was determined using a solution based on the low-affinity Ca^{2+} buffer nitrilotriacetic acid (NTA): 10 mM NTA: 3 mM Ca (yielding 50 μM free Ca using published K^d values). When using Mag-INDO-1, Mg^{2+} was omitted from all solutions (bath and intracellular).

Intensities are expressed in effectively absorbed photons, calibrated, as described previously, with respect to WT photoreceptors by counting quantum bump rates in response to the dimmest stimuli and measuring the relative intensities of all other stimuli (Hardie, 1995).

RESULTS

Controls

A number of control experiments were performed to establish that the indicator dye did not affect the light response, that the resting cytosolic Ca^{2+} concentration was stable over the time course of the experiments, and that the measuring flash did not necessarily permanently damage the cells. Ca^{2+} indicator dyes are themselves Ca^{2+} buffers and may, in principle, interfere with the kinetics of transduction, which are known to be very sensitive to cytosolic Ca^{2+} levels (Hardie, 1991). Figure 1 shows light responses made with control solutions containing no Ca^{2+} buffer and the solutions used for loading Ca^{2+} dyes into the cell. The responses were essentially indistinguishable, confirming that the concentration of dye used (100 μM) did not significantly affect the response to light under these conditions. Figure 1*b* also shows responses recorded before and after a measurement of cytosolic Ca^{2+} using a 200 msec measuring flash of submaximal intensity (containing 1.5×10^7 effective photons). Both the physiological response and the resting Ca^{2+} level (not shown) completely recovered within ~2 min. If the brightest intensities (~10× brighter) were used, WT photoreceptors almost invariably failed to recover sensitivity after a single measuring flash (see also Peretz et al., 1994a). However, because responses to maximal and submaximal intensities were otherwise essentially similar, it was often preferred to make single measurements on any given cell using maximum intensity to obtain superior signal-to-noise ratios.

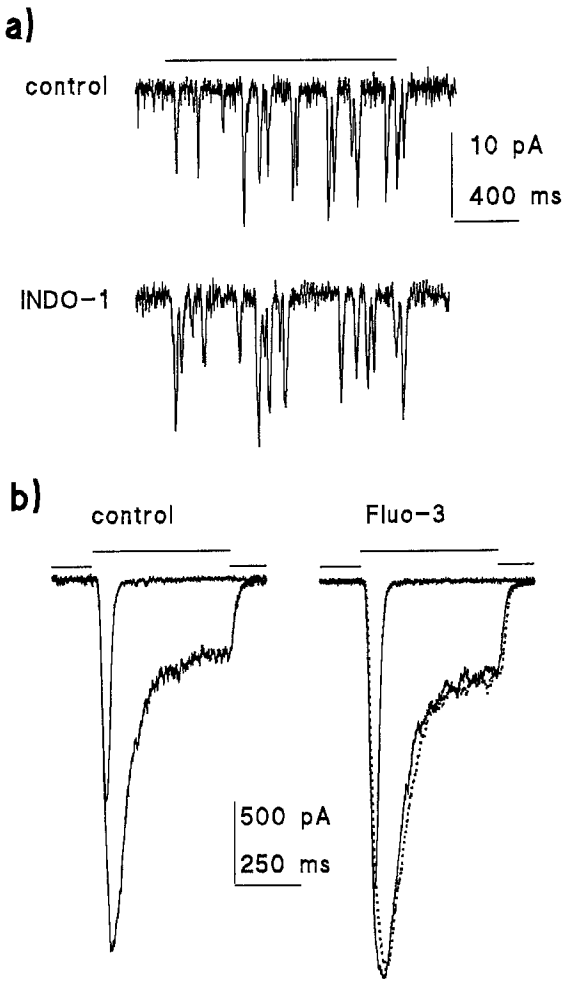

Figure 1. Indicator dyes do not significantly influence the light response. *a*, Quantum bumps recorded in response to a 1.5 sec stimulus of identical intensity in a cell loaded with 100 μM *INDO-1* are similar to those recorded from another cell with *control* intracellular solutions containing no Ca^{2+} buffers. *b*, Similarly, *Fluo-3* (100 μM) has little or no effect on responses to 20 msec flash or 500 msec step of light (the difference in absolute amplitudes is well within the experimental variability). The *dotted trace* shows the response to a second 500 msec stimulus recorded 2 min after making a Ca^{2+} measurement using a submaximal 100 msec flash containing $\sim 10^7$ effective photons. All responses recorded in standard Ringer's solution (1.5 mM Ca^{2+}) at a holding potential of −70 mV. Stimulus intensity in *b* was 3.8×10^4 effective photons · sec^{-1} (calibrated from bump counts as in *a*).

Figure 2 shows records of fluorescence measured from a photoreceptor of an *ora* mutant that completely lacked rhodopsin. Fluorescence was measured continuously, starting shortly after making the giga-seal. Immediately after establishing the whole-cell configuration (break-in), the fluorescence started to increase, indicating loading of the cell with dye. The time course of loading was usually well approximated by a single exponential with a time constant in the range of 30–80 sec. The Ca^{2+} concentration, determined from the ratio of fluorescence at 405 and 480 nm after subtraction of the background immediately before break-in, remained effectively constant during this period, showing only an improvement in the signal-to-noise ratio as the total dye concentration increases. However, immediately after break-in there was an indication that Ca^{2+} may actually decline over a period of ~10 sec; in addition, in some cells there was a slow rise in Ca^{2+}, typically starting after 3 or 4 min of whole-cell recording. To minimize variation attributable to the increases sometimes observed after longer periods of whole-cell recording, measurements in WT flies were routinely made between 2 and 3 min after break-in.

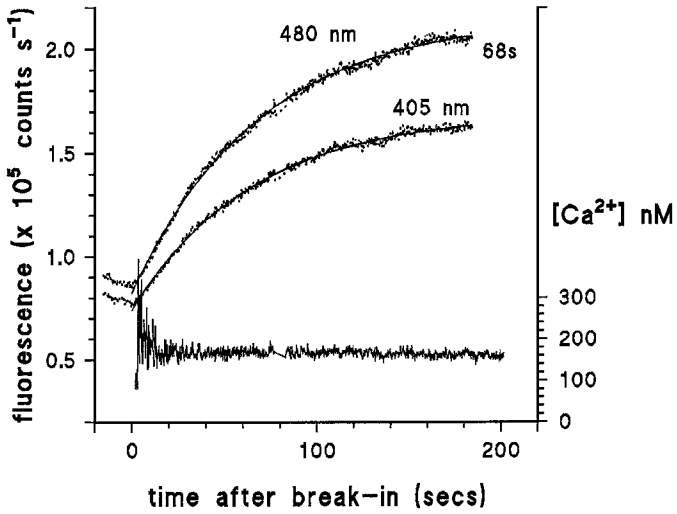

Figure 2. Time course of dye loading in an *ora* photoreceptor. Raw traces (*dotted*) show the INDO-1 fluorescence measured at 405 and 480 nm starting shortly after making a giga-seal. After establishing the whole-cell configuration (at time 0), fluorescence rises with a single exponential time course (*smooth curves*, single exponential fits to the traces with time constant of 68 sec). After subtraction of the background fluorescence, the ratio *R* between fluorescence at 405 and 480 nm was used to calculate cytosolic [Ca^{2+}] according to Equation 1. Cytosolic [Ca^{2+}] remained stable throughout the 200 sec recording, except for an apparent reduction during the first few seconds of recording. Recording made in normal (1.5 mM Ca^{2+}) Ringer's at −70 mV, briefly interrupted at ~80 sec, for visual inspection of cell.

Resting Ca^{2+} levels in the dark

Figure 3 shows a simultaneous recording of membrane current and INDO-1 fluorescence using 100 μM INDO-1 in a WT photoreceptor, sampled at 500 Hz. After a short latency (~10 msec), fluorescence measured at both wavelengths changed rapidly and in opposite directions (Fig. 3*a*) indicating a rise in Ca^{2+}. The time course of the calculated Ca^{2+} signal is shown below, along with the simultaneously recorded whole-cell current. The INDO-1 signal was rapidly saturated by the large Ca^{2+} increase (indicated by the increase in noise) so that, arguably, the most useful information to be gained from such records is the resting level determined during the latent period (Fig. 3*b*). When considering data only from "healthy" cells, using the criteria of negligible leak currents (<20 pA at a holding potential of −70 mV) and clearly discernible quantum bumps (5–10 pA) in response to weak illumination (e.g., Fig. 1), resting values showed rather little variation, averaging 161 ± 32 nM (n = 11) in the presence of normal Ringer's (1.5 mM). Resting values determined in WT flies were indistinguishable from values determined in *ora* mutants (162 ± 24 nM; n = 12), where resting levels could be more accurately determined, both because it is possible to record uninterrupted for several minutes and because the background level could be determined for each cell from the fluorescence before break-in (Fig. 2). The correspondence of values in WT and *ora* also indicate that the *ora* mutant may be usefully used for quantitative studies of factors affecting cytosolic Ca^{2+} other than light itself.

Identical measurements performed in the absence of extracellular Ca^{2+} (see further below) revealed a very significant drop in

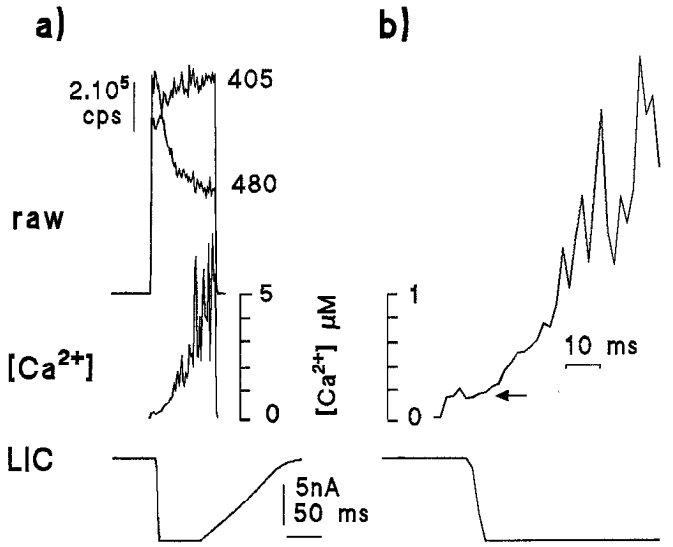

Figure 3. Light-induced Ca^{2+} rises measured with INDO-1 in normal (1.5 mM Ca^{2+}) Ringer's. *a*, Raw traces (*raw*) of fluorescence (counts per second) measured at 405 and 480 nm, sampled at 500 Hz; *[Ca^{2+}]*, after background subtraction, the time course of the rise in Ca^{2+} is calculated from the ratio of the raw traces according to Equation 1 (see Materials and Methods). The simultaneously recorded light-induced current (*LIC*, *lower trace*, holding potential −70 mV) saturated the amplifier (>10 nA). *b* shows the [Ca^{2+}] signal and LIC on an expanded scale. Notice that the dark level can be readily measured during the latent period of the response (*arrow*). The 150 msec UV stimulus (360 nm) contained 3×10^7 effective photons.

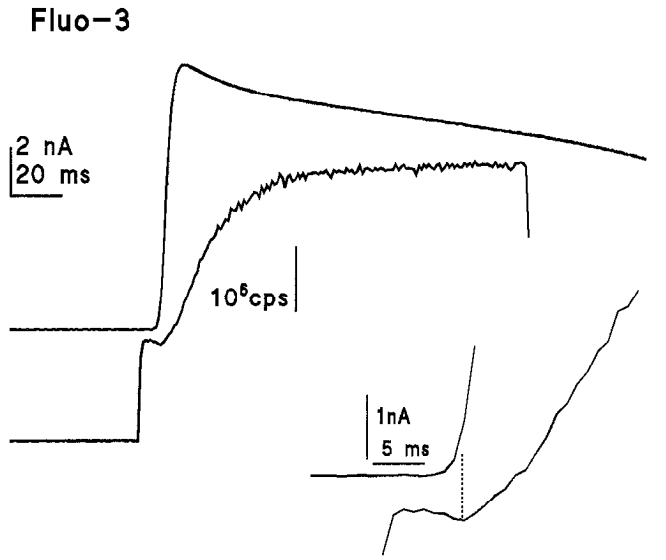

Figure 4. Latency of the Ca^{2+} response determined using Fluo-3 in normal Ringer's solution. The time course of the Ca^{2+} signal measured using the single-wavelength dye *Fluo-3* was similar to that measured with INDO-1 (compare Fig. 3); however, a better signal-to-noise ratio can be obtained. The traces show the average of LIC (*upper trace*) and Ca^{2+} signal from eight photoreceptors in response to a 200 msec measuring flash (480 nm) containing ~10^8 effective photons. The current trace has been inverted for easier comparison of time courses. The first detectable rise in Ca^{2+} always lagged the LIC by ~3 msec (*inset* on expanded scale). Holding potential was −70 mV, data sampled at 1 kHz.

resting cytosolic Ca^{2+}. In this case, however, values in *ora* mutants appeared somewhat lower than in WT (WT 70 ± 22 nM; n = 11; *ora* 28 nM ± 16; n = 36; measured at −70 mV), although, as emphasized in Materials and Methods, absolute levels in this range are prone to larger errors because of uncertainty of R_{min} values.

Latency

A number of studies have addressed the question of whether the Ca^{2+} signal measured in invertebrate photoreceptors precedes the electrophysiological response. For example, in *Limulus*, the light-induced Ca^{2+} signal, which is mainly attributable to release, was found to lag the electrical response by ~3–20 msec, thus calling into question the hypothesis that Ca^{2+} release is causal for excitation (Ukhanov et al., 1995) (see also Walz et al., 1994 for drone bee). In *Drosophila*, it now seems clear that virtually all the light-induced Ca^{2+} signal is attributable to Ca^{2+} influx (Peretz et al., 1994a; Ranganathan et al., 1994; see below), which should, in principle, coincide with the electrophysiological response. Measurements of latency in *Drosophila* thus provide an empirical test of the temporal resolution of whole-cell Ca^{2+} measurements. In practice, it was found that the best signal-to-noise ratio was found using the single-wavelength dye, Fluo-3, and this was preferred for obtaining information on latency, although there was no obvious difference from results using INDO-1. Figure 4 shows that even with the brightest intensities, there was always a significant delay between the light-induced current (LIC) and the first detectable rise in Ca^{2+}. Judged by eye, the average latency using the brightest intensity available was 9.1 ± 1.1 msec for the LIC and 11.9 ± 1.8 msec for the Ca^{2+} rise, or a lag of 2.8 ± 1.2 msec (n = 11 cells). The apparent lag increased considerably when lower intensities were used (e.g., a lag of 7.8 ± 2.3 msec; n = 10 with 10× lower intensity). Whatever the reasons for this lag (which presumably include the diffusional delay required for the Ca^{2+} to reach and bind to dye within a sufficient volume of the cell to overcome the measurement noise), these results indicate caution in interpreting temporal data derived from whole-cell fluorescent Ca^{2+} measurements. This problem appears now to have been resolved in *Limulus*, because recent measurements using confocal microscopy have shown that Ca^{2+} signals can indeed precede the electrical response (Ukhanov and Payne, 1995).

Quantification of light-induced Ca influx

Ca^{2+} rises elicited by weak illumination

To determine absolute Ca^{2+} levels reached in response to weak-to-moderate stimulation, a two-flash strategy was used similar to that recently used in *Limulus* ventral photoreceptors by Ukhanov et al. (1995). A 500 msec adapting step of light from a light-emitting diode (LED) of variable intensity was delivered to the cells, eliciting responses in the range of 0–2 nA in amplitude. Synchronously with the cessation of the adapting step, a brief (25–50 msec) UV measuring flash was applied, and the resting Ca^{2+} level was determined, as before, during the latent period (Fig. 5*a*). After pooling data from several cells, there was a clear intensity-dependent increase in the Ca^{2+} level. Because the Ca^{2+} increase derives almost exclusively from Ca^{2+} influx via the light-sensitive channels (Peretz et al., 1994a; Ranganathan et al., 1994; see further below), it seemed most instructive to plot the Ca^{2+} concentration reached as a function of the charge (time integral of current) carried by the response to the 500 msec adapting flash (Fig. 5*b*). A linear regression line plotted through the data indicated an increase of 2.7 nM for Ca^{2+} for each picocoulomb of current.

A second strategy for investigating the Ca^{2+} rises associated with small responses is to use mutants with greatly reduced rhodopsin levels, which consequently generate only small re-

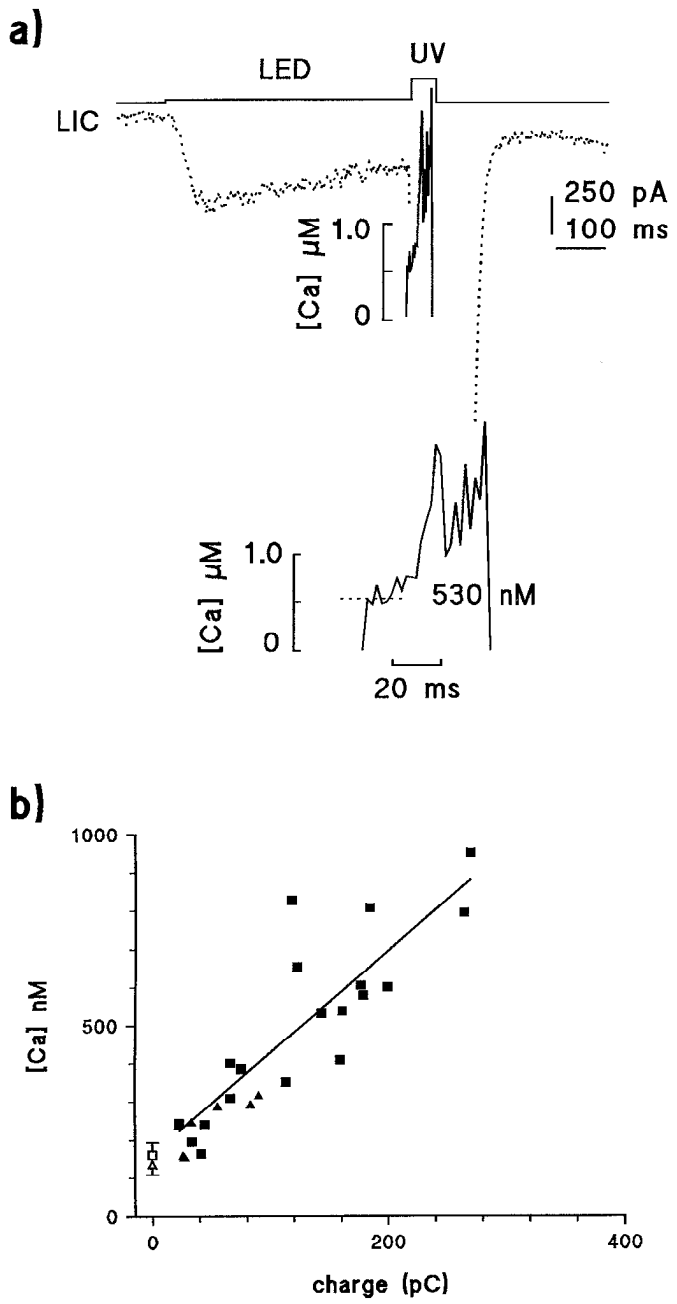

Figure 5. Determination of Ca^{2+} levels in response to weak illumination in normal Ringer's solution. *a*, A WT photoreceptor was first illuminated for 500 msec with a dim *LED* stimulus (~2000 photons/sec) generating an inward current of ~500 pA amplitude (*dotted trace*). The Ca^{2+} level reached during this period (530 nM) was then determined during the latent period of the response to a saturating UV measuring stimulus (50 msec, 3×10^7 effective photons). The Ca^{2+} signal (*solid trace*) is replotted on an expanded time base below. *b*, Ca^{2+} levels obtained from 19 cells (*filled squares*), as in Figure 5*a*, plotted against the total charge flowing during the 500 msec adapting step. *Open square*, "Dark" Ca^{2+} concentration determined identically, but without preillumination with the LED (mean ± SD of 12 cells). The data have been fitted by a regression line of slope 2.7 nM/pC with an intercept (dark resting Ca^{2+} level) of 161 nM. *Triangles* represent data determined using measurements of light-induced Ca^{2+} rises in *ora* or *ninaE* flies with small amounts of residual rhodopsin (Fig. 6).

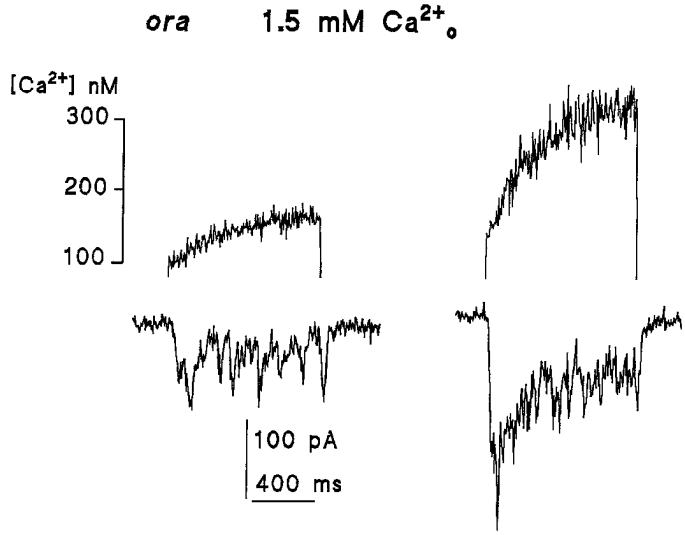

Figure 6. Ca^{2+} influx measured in real time in *ora* photoreceptors containing residual levels of rhodopsin. UV measuring flashes of 1 sec duration delivered to *ora* (or *ninaE*) photoreceptors sometimes elicited small responses (*lower traces*) attributable to residual levels of rhodopsin, thus allowing direct measurement of Ca^{2+} influx (*upper traces*) during weak effective illumination. Traces from two different cells are shown using different intensities, generating responses of ~20–40 pA (*left*) and 200 pA (*right*). Quantum bump noise can be clearly resolved in these small responses: as reported previously (Johnson and Pak, 1986), quantum bumps in *ninaE* mutants with greatly reduced rhodopsin levels were in fact typically larger than in WT. Substantial Ca^{2+} rises were detected in each case. The data from these and three other cells are plotted on Figure 5*b* and show reasonable agreement with measurements made in WT photoreceptors using the two-flash paradigm.

sponses to the bright measuring light. As reported previously (Johnson and Pak, 1986), there are a number of alleles of the rhodopsin gene *ninaE* in which the rhodopsin concentration in the photoreceptors is drastically reduced. The residual response to light in these mutants was apparently normal except that, as reported previously (Johnson and Pak, 1986), the quantum bumps were somewhat larger than in WT. Initially, the $ninaE^{P334}$ allele was tested, as photoreceptors in this mutant were reported to contain ~500 rhodopsin molecules (Johnson and Pak, 1986). However, photoreceptors from newly eclosed $ninaE^{P334}$ adults were found to be virtually unresponsive to the brightest stimuli available (equivalent to ~3×10^8 photons/sec in WT flies), indicating that, at this age at least, there can be at most only one or two rhodopsin molecules per cell. Surprisingly, it was found that photoreceptors from both the *ora* mutant and the $ninaE^{117}$ alleles, although reported to be completely null alleles, often produced responses up to ~200 pA in response to the brightest stimuli. Assuming a rhodopsin content of 10^8 molecules per WT cell (Johnson and Pak, 1986), the reduction in sensitivity in such cells would indicate a rhodopsin content of ~25 functional molecules. Figure 6 confirms that responses of ~20 pA are sufficient to give clearly resolvable rises in Ca^{2+} in these mutants. When plotted in the same way as the data collected from WT (i.e., as a function of charge carried), there was reasonable agreement in the absolute levels reached (Fig. 5*b*).

Maximum rises

The Ca^{2+} influx occurring during the response to the measuring stimulus clearly saturates high-affinity Ca^{2+} indicator dyes such as INDO-1 and Fluo-3 (see also Peretz et al., 1994a; Ukhanov et al., 1995). To gain an estimate of the absolute level of Ca^{2+} reached during more intense illumination, lower-affinity indicator dyes must be used. Currently, the only appropriate dual-emission dye

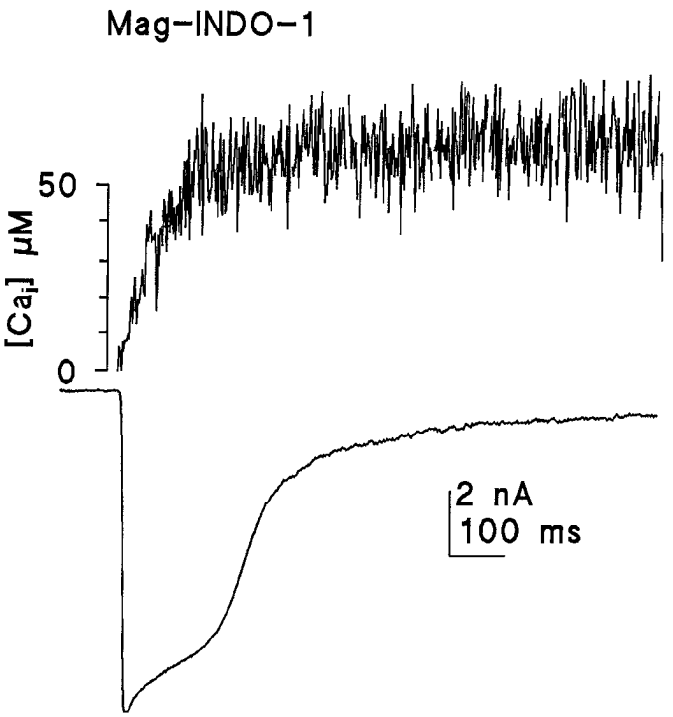

Figure 7. Light-induced Ca^{2+} rise in a WT photoreceptor loaded with the low-affinity indicator dye *Mag-INDO-1*. In response to a saturating UV stimulus, Ca^{2+} rose rapidly beyond 50 μM. Data were recorded at a holding potential of −70 mV in standard (1.5 mM Ca^{2+}) Ringer's solution containing no Mg^{2+}. Mg^{2+} was also omitted from the recording electrode solution. Similar results were obtained in four other cells.

available for this purpose is Mag-INDO-1. As implied by its name, this is actually designed as a Mg^{2+} indicator, but has an affinity for Ca^{2+} of ~100 μM and can be used for measuring Ca^{2+} in this range in the absence of Mg^{2+}. Cells were loaded with 100 μM Mag-INDO-1 using an electrode solution containing no Mg^{2+} and stimulated in Mg^{2+}-free external solutions. Under these conditions, Ca^{2+} increases to values >50 μM were observed (45 ± 23 μM; n = 4; e.g., Fig. 7).

Light-induced rise in *trp*

In the *trp* mutant, measurements of reversal potential have indicated that the Ca^{2+} permeability of the light-sensitive channels is severely reduced, leading to the proposal that the *trp* gene encodes a channel subunit responsible for the high Ca^{2+} selectivity of the light-sensitive channels (Hardie and Minke, 1992). To obtain independent confirmation of the reduced Ca^{2+} permeability of the light-sensitive channels in *trp*, Ca^{2+} influx in the *trp* mutant was quantified, as for WT, as a function of charge carried in the presence of normal (1.5 mM) extracellular Ca^{2+} using an identical twin-flash paradigm (as in Fig. 5). When quantified in this way, Ca^{2+} influx in the *trp* mutant was found to be reduced by a factor of ~2.5, now showing an increase of only 1.09 nM/pC (Fig. 8). By contrast, the resting Ca^{2+} concentration in the dark (i.e., during the latent period of the response to the measuring stimulus *without* preillumination) was not found to differ significantly from WT (153 ± 21 nM; n = 12; see Fig. 8).

Light-induced Ca^{2+} increase in the absence of extracellular Ca^{2+}

Previous studies reported either that there was no detectable light-induced Ca^{2+} rise in the absence of extracellular Ca^{2+}

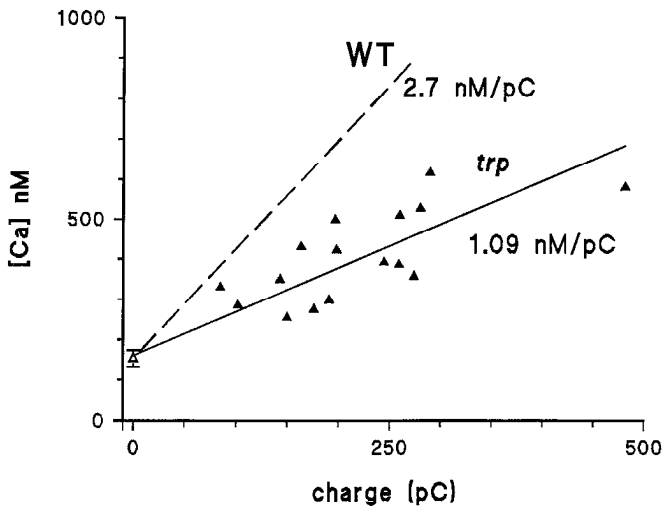

Figure 8. Ca^{2+} influx is reduced in the *trp* mutant. Measurements of Ca^{2+} levels in the *trp* mutant were made using the two-flash paradigm (Fig. 5) and plotted against the total charge carried in response to the adapting flash. In the dark (*open triangle*), resting Ca^{2+} levels in *trp* were indistinguishable from WT; however, in response to the 500 msec LED-adapting flash, the Ca^{2+} rise was significantly less than in WT (*dotted line* replotted from Fig. 5). The regression line through the *trp* data had a slope of 1.09 nM/pC (i.e., ~2.5× less than in WT).

(Ranganathan et al., 1994) or a very small rise that might have been attributable to influx of residual extracellular Ca^{2+} (Peretz et al., 1994a). Because of the importance of Ca^{2+} release for models of excitation, these experiments have been repeated and extended (see also Hardie, in press). Figure 9 shows the response of a cell exposed to Ca^{2+}-free Ringer's solution (2 mM EGTA, no added Ca^{2+}, and 120 mM NaCl). In contrast to previous results (Ranganathan et al., 1994), the light response was associated with a readily detectable Ca^{2+} signal increasing from a resting level of

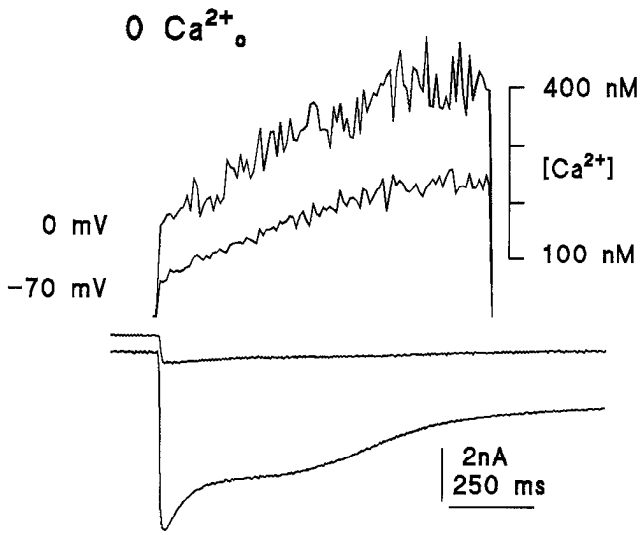

Figure 9. Light-induced Ca^{2+} signals measured in the absence of extracellular Ca^{2+}. Substantial Ca^{2+} increases were detected in every cell in response to saturating UV-measuring stimuli: Ca^{2+} signals (*upper traces*), simultaneously recorded whole-cell currents at 0 and −70 mV (*lower traces*) (two different cells). The rise was at least as large in cells clamped at 0 mV as in those clamped at −70 mV, arguing against influx of residual Ca^{2+} as an explanation. Note also that the initial (dark) resting level of Ca^{2+} was higher in the cell clamped at 0 mV (see also Fig. 11). Bath contained 0 Ca^{2+}, 2 mM EGTA, and 120 mM NaCl.

~70 to ~250 nM during a 1 sec saturating stimulus. It seems unlikely that this signal was attributable to influx of residual extracellular Ca^{2+}, because similar signals were seen in each of 15 cells tested even after exposure to the Ca^{2+}-free bath for 50 min (mean rise at a holding potential of −70 or −50 mV: 213 ± 125 nM; n = 11). In addition, the increase in Ca^{2+} was, if anything, increased (246 ± 146 nM; n = 4) when measurements were made at a holding potential of −10 or 0 mV, which would have substantially reduced the driving force for influx (Figs. 9, 11).

Initially these results appear to indicate the light-induced release of Ca^{2+} from intracellular stores; however, an alternative explanation may also be suggested. *Drosophila* photoreceptors have a powerful Na/Ca exchange mechanism (Hardie, 1995), which is probably one of the major mechanisms for maintaining low levels of intracellular Ca^{2+}. The response to light is inevitably associated with a large influx of Na^+ ions through the light-sensitive channels, which should reduce the Na^+ gradient available for Ca^{2+} extrusion. Assuming there is some internal Ca^{2+} source (e.g., the electrode solution, which was calculated to have a weakly buffered free Ca^{2+} concentration in excess of 200 nM or tonic flux from intracellular sources of Ca^{2+} such as the mitochondria), the resulting shift in the Na/Ca exchange equilibrium would be expected to result in a rise in cytosolic Ca^{2+}. To test whether such a mechanism might underlie the light-induced Ca^{2+} rises, extracellular Na^+ was replaced with NMDG to block the exchanger. To minimize the possibility of any long-term effects of this substitution, the NMDG solution was applied by rapid perfusion from a puffer pipette placed close to the recorded cell; as shown in Figure 10*c*, control responses recovered immediately after return to the original bathing solution. Under these conditions, the light-induced Ca^{2+} rise in Ca^{2+}-free Ringer's was virtually abolished, the average rise after 0.5 or 1 sec illumination now amounting to <20 nM (16 nM ± 13; n = 14; Figs. 10, 11). Although this is significantly ($p < 0.005$) different from zero, it is of the same order as the SD of the noise in these recordings, as well as the light-induced change in autofluorescence (which was subtracted from the raw data; see Materials and Methods), and it seems debatable whether it can be attributed to release.

The dark resting level of Ca^{2+} was also significantly increased by the NMDG substitution (from 70 ± 22 nM to 158 ± 43 nM; Fig. 11): this suggests that the Na/Ca exchange is indeed important in maintaining low Ca_i in Ca^{2+}-free solutions; however, it also raises the possibility that the block of the light-induced Ca^{2+} rise might have been attributable to inhibition by the raised resting Ca^{2+}. This possibility can probably be excluded because in the control (0 Ca, 120 mM NaCl) solution, depolarization caused a similar rise in the resting Ca^{2+} level (probably because of the voltage dependence of the Na/Ca exchange equilibrium), yet did not inhibit the subsequent light-induced increase (Figs. 9, 11).

Finally, the possibility was considered that the inability to detect significant release from stores was because of the putative light-sensitive stores becoming depleted during prolonged exposure to Ca^{2+}-free solutions (Hardie and Minke, 1992; Ranganathan et al., 1994). Therefore, measurements were also performed on photoreceptors bathed initially in normal (1.5 mM Ca^{2+}, 120 mM Na^+) Ringer's solution, but again briefly exposed to the same Ca^{2+}-free NMDG solution between 10 and 30 sec before making the measurements (Fig. 10*b*). Again, only a minimal and arguably insignificant Ca^{2+} rise was detected under these conditions (14 ± 8 nM; n = 8).

In summary, light-induced Ca^{2+} rises can indeed be detected in the absence of extracellular Ca^{2+}; however, the most likely explanation for this rise appears to be a shift in the Na/Ca exchange equilibrium. In the absence of external Na^+, the maximal rise detected is <20 nM over a time scale of 500 msec to 1 sec.

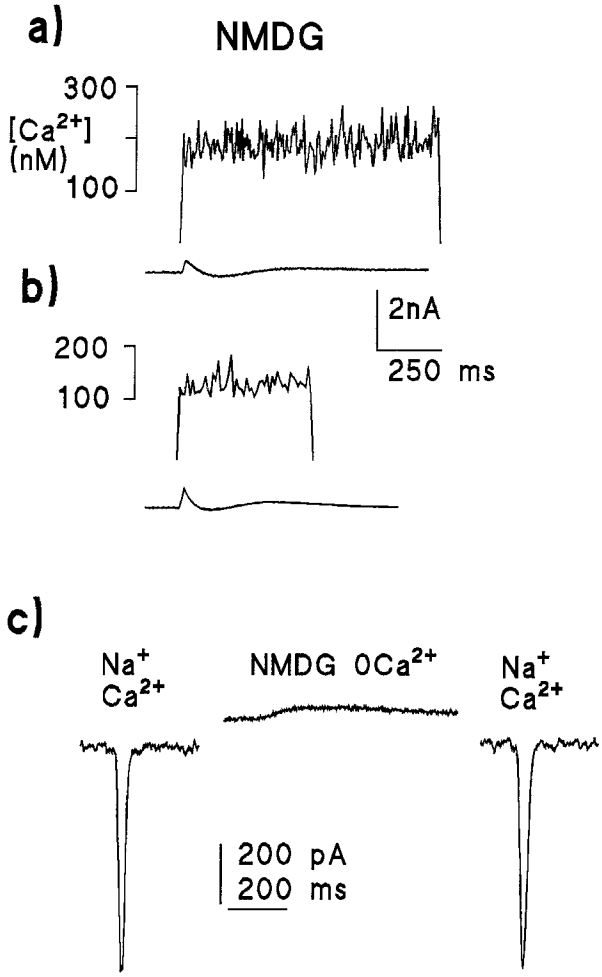

Figure 10. Light-induced Ca^{2+} signals and simultaneously recorded whole-cell currents (*lower traces*) measured in the absence of both extracellular Ca^{2+} and Na^+. In contrast to Figure 9, the Ca^{2+} rise was virtually abolished when Na^+ was substituted with *N*-methyl-D-glucamine (*NMDG*) with 0 Ca^{2+} and 2 mM EGTA applied by rapid perfusion from a puffer pipette. *a*, Response to a 1 sec saturating illumination in a cell perfused with NMDG solution, originally bathed in Ca^{2+}-free, Na^+-containing bath. *b*, Response from a photoreceptor perfused with NMDG, but this time after being initially bathed in normal Ringer's (1.5 mM Ca^{2+}); again there was little or no increase in Ca^{2+}. Both cells, clamped at −70 mV, produced small outward currents, as NMDG does not permeate the light-sensitive channels; *c*, Light-induced responses from the same cell as in Figure 11*b* to a weak 20 msec LED flash before (*left*), after (*right*), and during (*middle*) rapid perfusion with the Ca^{2+}-free NMDG solution. The cell was clamped at −70 mV. After perfusion with NMDG, the response reversed and became slower (because of the absence of Ca^{2+}-dependent feedback effects), but recovered completely after returning to normal Ringer's. The Ca^{2+} measurement was made ~60 sec after these responses were recorded, after the cell had been perfused again with NMDG for ~30 sec.

DISCUSSION

This study provides data on absolute levels of cytosolic Ca^{2+} in *Drosophila* photoreceptors and describes procedures for quantification of both dark- and light-induced Ca^{2+} signals in this important genetic model of phototransduction and PI signaling. The results confirm that the majority of the light-induced Ca^{2+} signal

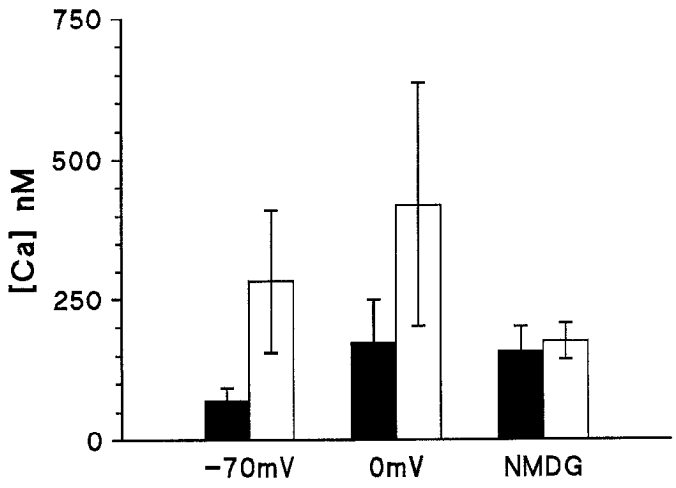

Figure 11. Summary of data collected in Ca^{2+}-free solutions. *Solid bars*, Dark levels determined during the initial 20 msec of the response; *open bars*, levels reached after 500 msec. In the presence of external Na^+, light-induced Ca^{2+} increases of >200 nM at both −70 mV (n = 11) and 0 mV (n = 4); note also that the depolarization alone increased the dark resting level from ~70 nM to 150 nM. After substitution with NMDG, the dark level also rose to ~150 nM; however, light now resulted in a barely significant rise of 16 nM. Data in NMDG, 0 Ca^{2+} solutions (n = 14 cells) were pooled from cells bathed initially in 0 Ca or 1.5 mM Ca^{2+} and regardless of holding potential (−70, −50 or 0 mV), since none of these conditions appeared to affect the resting Ca^{2+} in the absence of Na^+.

is attributable to Ca^{2+} influx; however, contrary to previous reports, significant rises can also be detected in Ca^{2+}-free conditions, probably as a result of Na^+ influx influencing the Na/Ca exchange equilibrium. With certain assumptions, the results also allow a number of useful quantitative estimates to be made, including (1) the effect of the *trp* mutation on the fractional Ca^{2+} current through the light-sensitive channels; (2) the effective buffering capacity of the photoreceptors; and (3) an upper limit on the amount of Ca^{2+} that might be released from internal stores—a result that has some implications for the still unresolved mechanism of excitation.

Subcellular compartments

The supposed Ca^{2+} stores (submicrovillar cisternae, or SMC) in *Drosophila* abut directly against the base of the microvilli, defining a putative "transduction compartment" consisting of the microvilli and the subrhabdomeric space, which is an ~10–20 nm gap between the SMC and the base of the microvilli (Walz, 1982). There is some evidence to suggest that indicator dyes or other substances introduced into *Drosophila* photoreceptors by patch pipettes do not permeate readily into the microvilli or the subrhabdomeric region (Ranganathan et al., 1994) (see also Hardie, 1995). The present paper adds little new information on this issue, except to reveal that even small LICs, which derive from channels believed to open into the subrhabdomeric space, give rise to readily detectable Ca^{2+} signals (Figs. 5, 6). However, one should be aware of the possibility that the transduction compartment might at least partially exclude the dye; the implications for some of the conclusions of this study are discussed where appropriate below.

Resting levels

By calibrating *in situ*, using solutions with ionic strength similar to those used for the actual measurements, many of the uncertainties associated with calibration of Ca^{2+} indicators should have been avoided in the present study. There is, however, still some uncertainty as to whether the measured values are representative of those occurring *in vivo*. For example, there was an indication over the first few seconds after break-in that [Ca] actually fell from a higher level (Fig. 2). One explanation may be that the electrode solution contained virtually no Na^+, whereas the photoreceptors can be expected to contain a few mM Na^+, which would result in a different equilibrium of the Na/Ca exchanger. An alternative possibility is that equilibration of the contents of the cell with the electrode solution may change the effective K^d of the dye by, for example, a change in the "viscosity factor." The dark, resting cytosolic Ca^{2+} level was found to be very sensitive to extracellular Ca^{2+}, falling from 150 to 70 nM (30 nM in *ora*). Although not systematically explored, it seems likely that Na/Ca exchange may be one of the major mechanisms responsible, because in the absence of extracellular Ca^{2+}, cytosolic Ca^{2+} rose to ~150 nM when extracellular Na^+ was substituted for NMDG (Fig. 11). A similar effect was elicited by depolarization, which is also predicted to shift the Na/Ca exchange equilibrium because of its voltage dependence.

Light-induced influx and buffering capacity

Measurements with Mag-INDO-1 indicated that Ca^{2+} may rise globally to levels as high as 50 μM; however, some caution should be exercised in accepting this figure. Recently, the effective single-channel conductance of the light-sensitive channels was found to increase ~10-fold in the absence of external Mg^{2+} (Hardie and Mojet, 1995). Therefore, under the Mg^{2+}-free conditions required to measure Ca^{2+} using Mag-INDO-1, the Ca^{2+} influx per channel is likely to be significantly greater. Conversely, this effect may be offset by the reduction in internal Mg^{2+} (omitted from the electrode solution), which results in an inhibition of the light response (R. Hardie, unpublished data). Nevertheless, the rapid saturation of INDO-1 signals (Fig. 2) clearly indicates that light-induced Ca^{2+} influx results in global concentration increases into the high micromolar range: locally, the levels are presumably even higher. These are unusually high Ca^{2+} loads and represent a considerable challenge for the Ca^{2+} homeostatic mechanisms of the photoreceptor.

For smaller currents, it was possible to quantify the Ca^{2+} rise more reliably (Fig. 5*b*). Comparison of this rise (2.7 nM/pC) with the predicted amount of Ca^{2+} influx via the light-sensitive channels provides an estimate of the effective buffering capacity of the photoreceptors, by which is understood here all mechanisms controlling cytosolic Ca^{2+}, including Ca-binding proteins, sequestration, extrusion by the Na/Ca exchanger or other transporters, and exclusion from the bulk of the cell by diffusion barriers. The relative permeability of the light-sensitive channels for Ca (P_{Ca}:P_{Na}) has been estimated at ~40:1 on the assumptions of the Goldman–Hodgkin–Katz (GHK) theory (Hardie and Minke, 1992). Solving the GHK constant current equation for the permeant ions suggests that ~49% of the current is carried by Ca^{2+} under the conditions of the experiment. Assuming the cell to be a cylinder 100 μm long and 5 μm in diameter, this should in fact raise global Ca^{2+} concentration by 1.29 μM/pC—suggesting that ~99.8% of the Ca^{2+} influx is effectively buffered (effective buffering capacity of ~500:1). Although the assumptions of the GHK analysis (e.g., independent mobility of ions) may be violated by the light-sensitive channels, even assuming a 10-fold lower P_{Ca}:P_{Na} ratio, ~16% of the current would be carried by Ca^{2+}, yielding an effective buffering capacity of ~150:1. Both of these figures are larger than values reported in other cells (e.g., 25–100:1 in chro-

maffin cells; Zhou and Neher, 1993), but similar to an estimate in the *Limulus* ventral photoreceptor (O'Day and Gray-Keller, 1989), suggesting that invertebrate photoreceptors may have evolved particularly powerful mechanisms for controlling cytosolic Ca^{2+} levels.

This calculation ignores the possibility that a component of the measured LIC is attributable to an electrogenic inward Na/Ca exchange current evoked by the Ca^{2+} influx. Although some contribution cannot be excluded, it is likely to be minor for the small currents used to determine the relationship between Ca_i and the influx current (Fig. 5*b*), because direct measurements of the Na/Ca exchange current using caged Ca^{2+} (Hardie, 1995) indicated that the exchange currents elicited by raising Ca^{2+} to 1 μM (the maximum level reached in Fig. 5*b*) were barely measurable (~4 pA).

Effect of the *trp* mutation on Ca^{2+} influx

Measurements of reversal potential indicated that the light-sensitive channels in the *trp* mutant have a reduced Ca^{2+} permeability (Hardie and Minke, 1992), suggesting that the *trp* gene encodes a light-sensitive channel subunit. Independent support for this important conclusion was provided by Peretz et al. (1994a,b), who showed that Ca^{2+} influx in response to bright lights in *trp* was substantially reduced. However, because the currents evoked in *trp* were also smaller, this result may have been a consequence of fewer channels being activated rather than a reduction in Ca^{2+} permeability per se. On the assumption that Ca^{2+} influx in WT and the *trp* has access to the same subcellular compartment(s) and is subject to the same buffering conditions, the relative fractional Ca^{2+} current through the light-sensitive channels in WT and *trp* should be given by the difference in slope of the plot of [Ca] versus charge, i.e., 2.7 nM/pC (WT): 1.09 nM/pC (*trp*), yielding an ~2.5× greater fractional Ca^{2+} current in WT. This estimate compares favorably with the fractional current predicted via GHK analysis [49% fractional Ca^{2+} current in WT compared with 13% in *trp*, assuming permeability ratios from Hardie and Minke (1992)]. In conclusion, on the assumption that Ca^{2+} influx is buffered equivalently in WT and *trp*, the present results provide strong confirmation of a major reduction in Ca^{2+} permeability in the light-sensitive channels of *trp* photoreceptors.

The question of light-induced Ca^{2+} release: implications for transduction

Contrary to previous reports (Peretz et al., 1994a; Ranganathan et al., 1994), in the present study substantial rises in Ca^{2+} were reliably detected in Ca^{2+}-free solutions. Previous failure to detect significant rises may have been attributable to the dyes acting as Ca^{2+} buffers (both studies used higher dye concentrations) or [in the case of Ranganathan et al. (1994)] less intense stimulation, or because measurements were always made from an already partially stimulated condition. Although the present results initially seemed to indicate substantial release of Ca^{2+} from internal stores, when Na^+ was replaced with NMDG, the light-induced Ca^{2+} rise was virtually abolished, leaving a residual signal of <20 nM. This suggests that most of the measured rise was not attributable to release from internal stores, but was probably attributable to a shift in the Na/Ca exchange equilibrium caused by Na^+ influx.

It is of some interest to inquire how much Ca^{2+} would be required to generate an increase of 20 nM, as this is probably an upper estimate of how much Ca^{2+} is released by a saturating light stimulus. In turn, this could provide clues whether such release might be sufficient to account for excitation—as demanded by some hypotheses of excitation (Shin et al., 1993). If it is assumed that any Ca^{2+} release is buffered and detected with the same efficiency as Ca^{2+} influx, then the Ca^{2+} flux required to generate a signal of 20 nM can be taken from the slope of the regression line for Ca^{2+} influx (2.7 nM/pC), i.e., ~7 pC for a 20 nM increase. Assuming 49% of the LIC is carried by Ca^{2+}, this would represent a pure Ca^{2+} current of 3.5 pC, corresponding, e.g., to 7 pA flowing for 500 msec, which is the typical duration of a quantum bump in Ca^{2+}-free Ringer's solution. This figure can be converted into numbers of $InsP_3$ receptors by assuming a value for their single-channel conductance. Bezprovanny and Ehrlich (1994) suggest a value of 0.5 pA under physiological conditions; however, this may be ~10× too high because it assumes a luminal Ca^{2+} concentration of 2.5 mM, whereas recent measurements suggest values closer to 200 μM (Hofer et al., 1995). Using a figure of 0.04 pA, the estimated Ca^{2+} flux required to generate a 20 nM rise would correspond to only 175 $InsP_3$ receptors being open on average for 500 msec.

By comparison, one can ask how many $InsP_3$ receptors might be required for excitation. As in most invertebrate photoreceptors, each effectively absorbed photon in *Drosophila* evokes a discrete quantum bump (Wu and Pak, 1975). The UV excitation light typically generated responses of >10 nA (underestimated because of imperfect voltage-clamp control). In Ca^{2+}-free Ringer's, quantum bumps are ~2 pA in amplitude (R. Hardie and S. Henderson, unpublished data) so that at least 5000 are likely to have contributed to the response. This large mismatch in the number of elementary excitatory events contributing to the response and the estimated number of $InsP_3$ receptors that one might expect to have been recruited (175) provides little support for the hypothesis that Ca^{2+} release via $InsP_3$ receptors is causal for excitation in *Drosophila* photoreceptors.

It should be emphasized that the above arguments assume that Ca^{2+} influx and Ca^{2+} release are buffered by the cell and detected by the indicator dye with similar efficiency. The possibility must also be considered that significant amounts of Ca^{2+} *are* released, but into a subcellular region distinct from that accessed by the light-sensitive channels and more remote from the indicator dye (assuming this to be at least partially excluded from the "transduction compartment"). This possibility cannot be excluded, but would contravene the widely accepted view that the putative release channels and influx channels both have access to the same compartment, i.e., the subrhabdomeric space between the SMC and the base of the microvilli.

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