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Calcium signalling in *Drosophila* photoreceptors measured with GCaMP6f

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ABSTRACT

Drosophila phototransduction is mediated by phospholipase C leading to activation of cation channels (TRP and TRPL) in the 30000 microvilli forming the light-absorbing rhabdomere. The channels mediate massive Ca^{2+} influx in response to light, but whether Ca^{2+} is released from internal stores remains controversial. We generated flies expressing GCaMP6f in their photoreceptors and measured Ca^{2+} signals from dissociated cells, as well as *in vivo* by imaging rhabdomeres in intact flies. In response to brief flashes, GCaMP6f signals had latencies of 10–25 ms, reached 50% F_{max} with ~1200 effectively absorbed photons and saturated ($\Delta F/F_0 \sim 10$–20) with 10000–30000 photons. In Ca^{2+} free bath, smaller ($\Delta F/F_0$ ~4), long latency (~200 ms) light-induced Ca^{2+} rises were still detectable. These were unaffected in $InsP_3$ receptor mutants, but virtually eliminated when Na^+ was also omitted from the bath, or in *trpl;trp* mutants lacking light-sensitive channels. Ca^{2+} free rises were also eliminated in Na^+/Ca^{2+} exchanger mutants, but greatly accelerated in flies over-expressing the exchanger. These results show that Ca^{2+} free rises are strictly dependent on Na^+ influx and activity of the exchanger, suggesting they reflect re-equilibration of Na^+/Ca^{2+} exchange across plasma or intracellular membranes following massive Na^+ influx. Any tiny Ca^{2+} free rise remaining without exchanger activity was equivalent to <10 nM ($\Delta F/F_0$ ~0.1), and unlikely to play any role in phototransduction.

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1. Introduction

Phototransduction in *Drosophila* is mediated by a G-protein coupled phospholipase C (PLC) signalling cascade [1–3]. All key elements of the transduction cascade from the visual pigment rhodopsin (Rh1) to the "light-sensitive" channels are localised within ~30000 microvilli forming a light-guiding "rhabdomere". Absorption of a single photon by one rhodopsin molecule results in a discrete electrical event (quantum bump), believed to reflect activation of PLC and ion channels within just one microvillus. The macroscopic current response to brighter light is the summation of multiple quantum bumps generated stochastically by absorption of photons across the microvillar population [4,5]. There are two distinct light-sensitive channels in *Drosophila*: TRP (transient receptor potential), which is the prototypical and defining member of the TRP ion channel superfamily [6,7], and a homologue, TRP-like, or TRPL [8,9], both belonging to the TRPC subfamily. Although, both are cation channels permeable to Ca^{2+}, TRP, which dominates the light-induced current (LIC) is particularly selective for Ca^{2+} (P_{Ca}:P_{Na} ~50:1), whilst TRPL has a more modest P_{Ca}:P_{Na} of ~4:1 [10,11]. As well as mediating a major fraction of the LIC [12], Ca^{2+} influx via these channels plays critical positive and negative feedback roles at multiple downstream targets and is essential for rapid kinetics and light adaptation [13,14].

Measurements using fluorescent Ca^{2+} indicators in dissociated *Drosophila* photoreceptors reveal that the Ca^{2+} signal in response to blue excitation light is dominated by massive Ca^{2+} influx via the light-sensitive channels [15–17]. Studies in larger flies using low affinity indicators show that Ca^{2+} levels in the microvilli reach near mM levels *in vivo* [18], and modelling suggests similar levels are reached in *Drosophila* [12,19]. In Ca^{2+} free solutions there is a much smaller (submicromolar) and slower rise in fluorescence, the origin and role of which is controversial [17,20–22]. Because $InsP_3$ is presumably generated in large amounts in response to the blue excitation, $InsP_3$-induced Ca^{2+} release from internal stores would seem the obvious explanation. However, using the high affinity ratiometric indicator INDO-1, this Ca^{2+} free signal was reported to be unaffected in null mutants of the only $InsP_3$ receptor (IP_3R) gene in the *Drosophila* genome [21]. Challenging this, Kohn et al.

Abbreviations: TRP, transient receptor potential; $InsP_3$, inositol (1,4,5) trisphosphate; IP_3R, $InsP_3$ receptor; PLC, phospholipase C; DPP, deep pseudopupil; R, rhodopsin; M, metarhodopsin.

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[22] reported that the Ca^{2+} free rise measured using the genetically encoded indicator GCaMP6f was substantially reduced following RNAi knockdown of the IP_3R and proposed that $InsP_3$-induced Ca^{2+} release played a critical role in phototransduction.

In the present study, we generated flies expressing GCaMP6f [23] in R1-6 photoreceptors under direct control of the Rh1 (*ninaE*) promoter. We performed measurements in dissociated ommatidia allowing control of extracellular solutions, and also *in vivo* from completely intact flies by imaging the rhabdomeres in the "deep pseudopupil" (DPP) [24–26]. By using 2-pulse protocols we provide data on the time course and intensity dependence of Ca^{2+} signals *in vivo* in response to physiologically relevant stimuli. We paid particular attention to the origin of the Ca^{2+} rise under Ca^{2+} free conditions, and found that it was unaffected in IP_3R mutants, but strictly dependent upon both Na^+ influx and Na^+/Ca^{2+} exchanger activity. We conclude that any light-induced release from internal stores is minimal (<10 nM), slow, and unlikely to play any direct role in phototransduction.

2. Materials and methods

2.1. Flies

Flies (*Drosophila melanogaster*) were reared on standard medium [recipe in [27]] at 25 °C in a dark incubator. For dissociated ommatidia, newly eclosed (<2 h) adults were used; for *in vivo* deep pseudopupil measurements flies were 1–7 days old. GCaMP6f (cDNA obtained from Addgene) was cloned into the pCaSpeR4 vector which contains a mini-w^+ gene as transfection marker and the *ninaE* (*Rh1*) promoter that drives expression exclusively in photoreceptors R1-6. The final construct (*ninaE-GCaMP6f*) was injected into w^{1118} embryos and transformants recovered on 2nd and 3rd chromosomes. The *ninaE-GCaMP6f* transgene was crossed into various genetic backgrounds including:

trp^{343} – null mutant lacking TRP channels [28],

$trpl^{302}$ – null mutant lacking TRPL channels [9]

and $trpl^{302}$;trp^{343} – double null mutant lacking all light-sensitive channels.

$norpA^{P24}$ – null mutant of PLC [29].

$calx^1$ – severe hypomorphic mutant of the Na^+/Ca^{2+} exchanger (*calx*) with no detectable exchanger activity in the photoreceptors [30].

ninaE-calx/CyO – flies over-expressing a wild-type *calx* transgene under control of the Rh1-promoter [30].

($l(3)itpr^{90B.0}$ – larval lethal null mutant of $InsP_3$ receptor: referred to as *itpr* [31].

To generate *ninaE-GCaMP6f* in whole eye IP_3R null (*itpr*) mosaics:

ninaE-GCaMP6f/Cy;FRT82B, ($l(3)itpr^{90B.0}$/*TM6:* were crossed to *yw;P{w+, ey-Gal4,UAS-FLP}/CyO;P{ry+,FRT82B}P{w+ GMR-hid},3CLR/TM6* – Bloomington stock 5253. Non-*Cy* and non-*TM6* F1 then have *itpr* homozygote null mosaic eyes and *ninaE-GCaMP6f* [21,32].

2.2. Electrophysiology

Whole-cell patch clamp recordings of photoreceptors from dissociated ommatidia from newly eclosed adult flies of either sex were performed as previously described [e.g. 33] on an inverted Nikon microscope (Nikon UK). Standard bath contained (in mM): 120 NaCl, 5 KCl, 10 *N*-Tris-(hydroxymethyl)-methyl-2-aminoethanesulphonic acid (TES), 4 $MgCl_2$, 1.5 $CaCl_2$, 25 proline and 5 alanine, pH 7.15. For Ca^{2+} free bath $CaCl_2$ was omitted and 1 mM Na_2EGTA added. The intracellular pipette solution was (in mM): 140 K gluconate, 10 TES, 4 Mg-ATP, 2 $MgCl_2$, 1 NAD and 0.4 Na-GTP, pH 7.15. Chemicals were obtained from Sigma-Aldrich (Gillingham, UK). Recordings were made at room temperature (21 ± 1° C) at −70 mV (including correction for −10 mV junction potential) using electrodes of resistance 10–15 MΩ. Data were collected and analysed using an Axopatch 200 amplifier and pCLAMP v.9 or 10 software (Molecular Devices, Union City CA). Quantum bumps were analysed using Minianalysis software (Synaptosoft.com). Photoreceptors were stimulated via a green (522 nm) ultrabright light-emitting-diode (LED) controlled by a custom made LED driver; intensities were calibrated in terms of effectively absorbed photons by counting quantum bumps at low intensities.

2.3. GCaMP6f measurements

Fluorescence measurements were made as previously described [25,34] on an inverted Nikon microscope (non-confocal) from dissociated ommatidia or *in vivo* by imaging the DPP in intact flies immobilised with low melting point wax in truncated plastic pipette tips. Excitation light (470 nm) was delivered from a blue power LED (Cairn Research UK) and fluorescence observed using 515 nm dichroic and OG515 long-pass filters. Fluorescent images were sampled and analysed at up to 500 Hz using an Orca 4 camera and HCImagelive software (Hamamtsu); but for most experiments fluorescence of whole ommatidia (via 40 x oil objective), or DPP (20 x air objective) was directly measured via a photomultiplier tube (Cairn Research UK), sampled at up to 2 kHz and analysed with pCLAMP software. Background fluorescence was subtracted using estimates from identical measurements from flies lacking fluorescent constructs, but with similar eye colour (most autofluorescence derives from screening pigment granules). Following each measurement the ommatidium/fly was exposed to intense, photo-equilibrating red (4 s, 640 nm ultra-bright LED) illumination to reconvert metarhodopsin (M) to rhodopsin (R), and allowed to dark adapt for at least one minute before the next measurement.

For Ca^{2+} free measurements, dissociated ommatidia (plated in standard bath) were briefly perfused with a Ca^{2+} free solution (0 Ca^{2+}, 1 mM Na_2EGTA see 2.2) or a Na^+ and Ca^{2+} free solution in which NaCl was substituted for equimolar LiCl, KCl, CsCl or NMDGCl (1 mM K_2EGTA and 4 mM $MgCl_2$ also present). Ommatidia were individually perfused by a nearby (~20 μm) puffer pipette and measurements made within ~20–50 s of perfusion onset. Following M to R photoreconversion the cells were returned to normal (1.5 mM Ca^{2+}) bath and dark-adapted for at least three minutes before the next measurement.

For 2-pulse experiments, green light was supplied by a green (λ_{max} 522 nm) LED (for dissociated ommatidia) or for the DPP by a "warm-white" power LED (Cairn Research UK) filtered by a GG 475 filter (resulting λ_{max} 546 nm). The green illumination was calibrated in terms of effectively absorbed photons by counting quantum bumps in whole-cell recordings or, for *in vivo* measurements from the DPP, by measuring the rate at which it converted M to R spectrophotometrically in the same set up, as previously described [26].

2.4. GCaMP6f calibration

Maximum and minimum fluorescence of GCaMP6f *in situ* was calibrated by exposing dissociated ommatidia to ionomycin (10 μM) and then perfusing alternately for several minutes with 10 mM K_2EGTA 100 mM KCl 10 MOPS pH 7.2 (nominally 0 Ca^{2+}) and 10 mM CaEGTA 100 mM KCl 10 MOPS (nominally 40 μM Ca^{2+}) (solutions from Biotium Ca^{2+} calibration buffer kit). After background subtraction, $\Delta F/F_0$ with the saturating 40 μM Ca^{2+} solution (F_{max}) was 23.5 ± 1.52 (mean ± S.E.M. n = 8), which is close to the published *in vitro* value of 25 [35]. For estimating absolute cytosolic Ca^{2+} levels [Ca_i] from $\Delta F/F_0$ values, we assumed our F_{max} value

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