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Speed and Sensitivity of Phototransduction in *Drosophila* Depend on Degree of Saturation of Membrane Phospholipids

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*Drosophila* phototransduction is mediated via a G-protein-coupled PLC cascade. Recent evidence, including the demonstration that light evokes rapid contractions of the photoreceptors, suggested that the light-sensitive channels (TRP and TRPL) may be mechanically gated, together with protons released by PLC-mediated PIP2 hydrolysis. If mechanical gating is involved we predicted that the response to light should be influenced by altering the physical properties of the membrane. To achieve this, we used diet to manipulate the degree of saturation of membrane phospholipids. In flies reared on a yeast diet, lacking polyunsaturated fatty acids (PUFAs), mass spectrometry showed that the proportion of polyunsaturated phospholipids was sevenfold reduced (from 38 to ~5%) but rescued by adding a single species of PUFA (linolenic or linoleic acid) to the diet. Photoreceptors from yeast-reared flies showed a 2- to 3-fold increase in latency and time to peak of the light response, without affecting quantum bump waveform. In the absence of Ca2+ influx or in *trp* mutants expressing only TRPL channels, sensitivity to light was reduced up to ~10-fold by the yeast diet, and essentially abolished in hypomorphic G-protein mutants (*Gaq*). PLC activity appeared little affected by the yeast diet; however, light-induced contractions measured by atomic force microscopy or the activation of ectopic mechanosensitive gramicidin channels were also slowed ~2-fold. The results are consistent with mechanosensitive gating and provide a striking example of how dietary fatty acids can profoundly influence sensory performance in a classical G-protein-coupled signaling cascade.

Key words: lipidomics; mechanosensitivity; phosphoinositide; phospholipase C; photoreceptors; PIP2

Introduction

Phototransduction in *Drosophila* is mediated by a G-protein-coupled PLC cascade, localized in a stack of ~30,000 microvilli together forming a light-guiding rhabdomere. The electrical response is characterized by exquisite sensitivity to single photons with kinetics ~10–100× faster than in vertebrate rods (Yau and Hardie, 2009; for review, see Katz and Minke, 2009; Fain et al., 2010; Hardie, 2012). The response is mediated by Ca2+-permeable cation channels encoded by the *transient receptor potential* (*trp*) and *trp*-like (*trpl*) genes (Hardie and Minke, 1992; Phillips et al., 1992; Niemeyer et al., 1996; Reuss et al., 1997). Although *Drosophila* TRP was the prototypical member of the TRP ion channel family (Montell and Rubin, 1989; Hardie and Minke, 1992; Hardie, 2011), how TRP and TRPL, or their vertebrate TRPC homologs, are activated downstream of PLC remains controversial (for review, see Katz and Minke, 2009; Hardie, 2012; Montell, 2012). PLC hydrolyzes the membrane phospholipid, phosphatidylinositol 4,5 bisphosphate (PIP2), generating DAG and inositol 1,4,5 trisphosphate (InsP3). In general, neither DAG nor InsP3 are effective activators of the light-sensitive channels when applied exogenously (for review, see Raghu and Hardie, 2009; Hardie, 2012); although slow activation of TRP channels by DAG was recently reported in excised patches (Delgado and Bacigalupo, 2009; Delgado et al., 2014). In contrast, both TRP and TRPL can be rapidly activated by polyunsaturated fatty acids (PUFAs; Chyb et al., 1999; Lev et al., 2012); however, a lipase that could release PUFAs from DAG in response to light has not been identified (Leung et al., 2008).

Two additional consequences of PLC activity have recently been implicated in channel activation. First, PLC reduces PIP2 levels, and second, it releases protons. Strikingly, the strict combination of PIP2 depletion and acidification rapidly and reversibly activates native TRP and TRPL channels (Huang et al., 2010). This might suggest that PIP2 binds to the channels, and when PIP2 dissociates, it exposes a protonatable site, which mediates activation by protons. However, evidence suggests that PIP2 de-

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pletion might also act in a mechanical sense; namely, by removing its bulky inositol headgroup, hydrolysis of PIPI₂ directly affects the physical properties of the lipid bilayer. Recently a direct manifestation of this was observed as rapid light-evoked contractions of photoreceptors (Hardie and Franze, 2012). Involvement of membrane physical properties in channel activation was also described previously (Parnas et al., 2009).

If mechanical gating is involved, we predicted that the response to light should be influenced by altering the physical properties of the membrane. To this end we used diet to manipulate the degree of saturation of membrane phospholipids—a key factor controlling membrane stiffness and elasticity (Brenner, 1984; Rawicz et al., 2000). Strikingly, increasing the proportion of saturated phospholipids resulted in a 2- to 3-fold increase in response latency, which appeared to be mediated downstream of PLC and was associated with a slowing of the light-induced contractions. The results are consistent with mechanical gating and provide a striking demonstration of how dietary fatty acids can influence a defined step in a classical G-protein-coupled signaling cascade.

Materials and Methods

Flying (Drosophila melanogaster) were reared on various diets (see below) in the dark at 25°C. The wild-type strain was Oregon; for some experiments white-eyed mutants (w¹¹⁸ or cn, bw) were used, which are indistinguishable in terms of whole-cell electrophysiology. Mutants used included: w¹¹⁸; trp;²⁴, a null mutant of the major light-sensitive TRP channel (Scott et al., 1997); cn, bw, trp;²⁰, a null mutant of the TRPL channel (Niemeyer et al., 1996); and cn, bw, Goa⁴, a severe hypomorph of the G-protein subunit, expressing ~1% functional protein (Scott et al., 1995). For pH microfluorometry we used pRh3.1; ninaE⁸ flies (Wardill et al., 2012) expressing the UV rhodopsin Rh3 on a ninaE⁸ background (near null mutant for the normal Rh1 opsin).

Food. The yeast-based diet (YE diet) consisted of the following: 100 ml of tap water, 1 g of agar, 8 g of baker’s yeast, 5 g of sucrose, 5 ml of nopaline (2.9%), and 25 mg of β-carotene. Flies were reared on either this YE diet or with the addition of 50 µl/100 ml of a single species of fatty acid: linoleic acid (LA; 18:2), linolenic acid (LNA; 18:3), oleic acid (OIL; 18:1), stearic acid (ST; 18:0), or palmitic acid (PAL; 16:0). For food preparation, tap water; agar, yeast, and sucrose were mixed at room temperature, before microwaving for 2 min. Nopaline, β-carotene, and fatty acids (when used) were added after cooling and mixed in using an electric blender. Control flies were also reared on standard plant/yeast-based medium (8.5 g of cornmeal, 0.9 g of agar, 1.5 g of yeast, 7.5 g of glucose, and 5 ml nopaline/100 ml water).

Electrophysiology. Whole-cell patch-clamp recordings of photoreceptors from dissociated ommatidia from newly eclosed adult flies of either sex were made as previously described (Hardie et al., 2002) on an inverted Nikon Eclipse TE300 microscope. Standard bath contained the following (in mM): 120 NaCl, 5 KCl, 10 N-Tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES), 4 MgCl₂, 1.5 CaCl₂, 25 proline, and 5 alanine, pH 7.15. The intracellular pipette solution was as follows (in mM): 140 K gluconate, 10 TES, 4 Mg-ATP, 2 MgCl₂, 1 NAD, and 0.4 Na-GTP, pH 7.15. Chemicals were obtained from Sigma-Aldrich. Recordings were made at room temperature (20 ± 1°C) at 70 mV (including correction for ~10 mV junction potential) using electrodes of resistance ~10–15 MΩ. Series compensation of ~80% was applied for macroscopic responses, but not for sampling quantum bumps and dark noise. Data were sampled at 0.5—2 kHz and filtered (4-pole Bessel) at 0.2—1 kHz using Axopatch 200 or 100 amplifiers and pClamp software (Molecular Devices). Quantum bumps and spontaneous dark events were analyzed from favorable recordings (baseline noise SD typically 0.3—0.5 pA) using Mini analysis (Jaejin Software), setting a peak threshold criterion of 0.5 pA and area threshold of 8—12 pA.ms for dark events and 2 pA and 40 pA.ms for quantum bumps, respectively. Unless otherwise stated, photoreceptors were stimulated via a green (540 nm) ultrabright LED; intensities were calibrated in terms of effectively absorbed photons by counting quantum bumps at low intensities. Because responses scale strictly linearly with intensity over a substantial range of intensities (Henderson et al., 2000), sensitivity within this linear range can be measured from response amplitudes to flashes of fixed intensities.

For pH microfluorometry, the fluorescent pH indicator 8-hydroxypyrrene-1,3,6-trisulfonic acid (HPTS; 500 µM) was loaded into photoreceptors via the patch electrode, allowing ~2—3 min for equilibration before making measurements as previously described (Huang et al., 2010). Excitation light (470 nm) was delivered from a high-power LED and fluorescence of the whole-cell measured via a photomultiplier tube (Cairn Research) using 515 nm dichroic and OG515 long-pass filters. Absolute pH shifts (∆pH) can be estimated using single wavelength fluorometry if the starting pH value (pHstart) is known (Schwiening and Willoughby, 2002):

\[
\Delta pH = \log(F/F_0) - \log(1 - F/F_0 - 1) \times 10^{(\text{pK}_a - \text{pHstart})}
\]

where F is the instantaneous fluorescence and F₀ is the fluorescence at the start of the recording. pKₐ for HPTS was taken as 7.18 (Schwiening and Willoughby, 2002) and pHstart 7.15 (pH of the bath and patch pipette solutions).

In vivo electrophysiology. Flies (1—10 d post-eclosion females) were kept in darkness at 19°C for at least 3 d before the experiments. For intracellular recordings, flies were immobilized in a brass fly holder with beeswax, as described previously (Juusola and Hardie, 2001). Conventional sharp microelectrodes (120—220 MΩ) were pulled from borosilicate glass with a Sutter Instruments P2000 puller, filled with 3 M KCl solution and inserted via a small hole cut in the dorsal cornea sealed with Vaseline. A blunt reference electrode, filled with fly ringer, was inserted into the head near the ocelli. The fly’s head was maintained at 20 ± 1°C by a feedback-controlled Peltier device. Recordings were performed using the discontinuous (switched) clamp method with a switching frequency of up to 40 kHz. The selected R1–R6 photoreceptors typically had resting potentials ~<−55 mV in darkness and ~40—60 mV responses to a saturating 10 ms light pulse (estimated to contain ~10⁵ photons). Photoreceptors were stimulated with a high-power LED (Seoul Z-Power LED P4 star, white, 100 lumens), driven by an Opto-LED (Cairn Research). The LED was connected to a randomized quartz fiber optic bundle, fitted with a lens and a pinhole (~2° as seen by the flies), centered on the photoreceptor’s receptive field using a Cardan arm device. Both the stimuli and the voltage responses were low-pass filtered (4-pole Bessel) at 500 Hz, and sampled at 1 kHz using a 12-bit A/D converter (National Instruments), controlled by a custom-written software system, Biosyst (Juusola and Hardie, 2001; Juusola and de Polavieja, 2003) in MATLAB (MathWorks).

The analytical and information theoretical methods used have been described in detail previously (Juusola et al., 1994; Juusola and Hardie, 2001; Juusola and de Polavieja, 2003), and are briefly summarized here. We selected a highly variable 1 s long naturalistic light-intensity time series sequence (NS; 10,000 points) from the van Hateren stimulus collection (van Hateren, 1997), with a power spectrum following −1/f statistics (where f = temporal frequency). The NS, with mean effective intensity of ~10⁻⁵ photons/s, was played back at 10 kHz and repeated 50—100 times, while continuously recording the photoreceptor’s voltage response. The first 5–20 traces showed short-term adaptive trends and were rejected from the analysis.

In each recording, the mean was the signal, and the noise was the difference between individual traces and the signal. Therefore, from n trials we obtained one signal trace and n noise traces. The signal and noise traces were divided into 50% overlapping stretches and windowed with a Blackman–Harris 4-term window, each giving three 500 point long samples. Twenty consecutive traces were selected from the most stable continuous segment in the recorded voltage responses, from which we obtained 60 spectral samples for the noise, and 3 spectral samples for the signal. These were averaged, respectively, to improve the estimates. Signal–to-noise ratio, SNR(F), estimates of the voltage responses were calculated from their signal and noise power spectra, |<S(f)|²> and |<N(f)|²>, respectively, as their ratio, where ∣ | denotes the norm and
Figure 1. Diet has only minor effects on visual pigment and morphology. A, Reflectance/scattered light measurements from deep pseudopupil of intact white-eyed fly (w1118); rhodopsin was first converted to metarhodopsin (M) by bright blue (BI = 470 ± 20 nm) excitation, and then reconverted to the rhodopsin state (R) by long wavelength green illumination (Gr = 550 ± 50 nm). Because M absorbs maximally 570 nm and R at 480 nm, less long wavelength light is absorbed by the eye as M is photoreisomerized back to R and the intensity of the reflected/back-scattered green light (S) increases with an exponential time constant reflecting the rate of M>R conversion (inset on expanded scale). The relative increase during this phase (ΔS/S) provides a measure of the concentration of visual pigment in the eye. B, Relative rhodopsin (Rh1) content in wild-type (wt), trpl, trp, and GoaQ mutants derived from ΔS/S values normalized to wild-type (w1118) control flies reared on normal diet. In most cases values for flies reared on both YF and LNA diets were reduced ~2-fold compared with control diet (white bars), but YF and LNA-reared flies in each genotype showed no significant differences from each other (one-way ANOVA). Mean ± SEM; n = 6–10 flies each. C, Dissociated ommatidia from flies reared on normal, YF, and LNA diets appeared similar (fluorescent micrographs of ommatidia from wild-type flies expressing GFP-tagged arrestin to highlight rhabdomeres). Scale bar, 10 μm. D, Whole-cell capacitances in wild-type, trpl, trp, and GoaQ flies reared on control, YF, and LNA diets (mean ± SEM; n = 8–27 cells each). In each genotype, values on YF and LNA diets were statistically indistinguishable (one-way ANOVA), although usually slightly lower than on control diet.

<> the average over the different stretches. Note that since SNR(f) estimates of YF. LNA control photoreceptor outputs were calculated using the same number of traces, and their comparison was immune to data-point bias.

To estimate information transfer rates of photoreceptor voltage responses, we used both the classic Shannon formula and the triple extrapolation method (Juusola and de Polavieja, 2003). But since both methods provided similar estimates (cf. Song and Juusola, 2014), showing consistent differences between the test (YF and LNA) and control groups, we show only the information rates, C, obtained from the Shannon formula (Fig. 5):

\[ C = \int_{\text{min}}^{\text{max}} \log_2(SNR(f)) + 1 \, df \]

where \( \text{min} = 2 \, \text{Hz} \) and \( \text{max} = 500 \, \text{Hz} \) (resulting from 1 kHz sampling rate and 300 point window size).

Electrorretinograms were recorded as described previously (Satoh et al., 2010) from flies of either sex immobilized with low melting point wax in truncated pipette tips using low resistance (~10 MΩ) glass microelectrodes filled with fly Ringer (140 mM NaCl, 5 KCl, 1.5 CaCl2, and 4 MgCl2), one inserted into the eye and one into the head capsule near the ocelli. Light from a 100 W halogen lamp was filtered by a Schott OG550 long-pass filter and glass neutral density filters and delivered to the eye by a fluid-filled light guide positioned within 5 mm of the fly’s head. Signals were amplified by a Neurolog NL102 DC (Digitimer) preamplifier and sampled and analyzed using pClamp software (Molecular Devices).

Measurements of Rhodopsin concentration. Fly metarhodopsin (M) is thermostable and absorbs maximally at ~570 nm, while the rhodopsin (R) state absorbs maximally at 480 nm. The M and R states are photo-interconvertible and exist in a photo-equilibrium, determined by the spectral content of illumination and the R and M photosensitivity spectra (Minke and Kirschfeld, 1979; Belusic et al., 2010). Long wavelength light reflected and scattered back out of the eye is more effectively absorbed by M than it is by R. Consequently, when delivered after first establishing a photo-equilibrium with blue light (generating ~70% M), the intensity of back-scattered long wavelength light increases as M is photoreisomerized back to R with a single exponential time course that provides a direct measure of the rate of photon absorptions by M. The increase in reflected/back-scattered light as M is reconverted to R provides a measure of the concentration of visual pigment and was used to compare visual pigment concentrations in flies reared on different diets (Fig. 1A).

Optomotor behavior experiments. For flight simulator experiments, we used 3- to 7- d-old female flies, reared in a 12 h dark/light cycle. Flies were tethered in a classic torque meter (Tang and Guo, 2001), with heads fixed, and lowered by a manipulator in the center of a black–white cyl-
inder (spectral full-width: 380–900 nm). A flying fly saw a continuous (360°) stripe scene. After viewing the still scene for 1 s, it was spun counterclockwise by a linear stepping motor for 2 s, stopped for 2 s before rotating clockwise for 2 s, and stopped again for 1 s. This 8 s stimulus was repeated 10 times and each trial, together with the fly’s yaw torque responses, was sampled at 1 kHz (Wardill et al., 2012). Flies followed the scene rotations, generating yaw torque responses (optomotor responses to right and left), the strength of which reflects the strength of their motion perception. Stimulus parameters for the moving stripe scenes were as follows: azimuth ± 360°, elevation ± 45°, wavelength 14°, and contrast 1.0, as seen by the fly. For slow scene rotation, the velocity was 45°/s and for fast rotation, 180°/s.

Atomic force microscopy. Atomic force microscopy (AFM) measurements were made as previously described (Hardie and Franz, 2012) from dissected retinas attached to glass slides using Cell-Tak Cell and Tissue Adhesive (BD Biosciences) in normal bath solution. Monodisperse polystyrene beads (37.28 ± 0.34 μm; microParticles) were glued to silicon cantilevers (Arrow TL1; NanoWorld; nominal spring constant: 0.03 N/m). The cantilevers were mounted on a JPK NanoWizard 3 AFM (JPK Instruments), placed on the same inverted microscope (Nikon Eclipse TE300) used for whole-cell patch-clamp measurements. The cantilever probe was approached onto the retina where it stayed in contact with the distal ends of a small group of photoreceptors, with a set force of 200 pN. Cantilever deflection was maintained constant and z-piezo position sampled at 2 kHz and synchronized to the triggered light pulses using pClamp software (Molecular Devices).

Lipidomics. For each sample 100–200 recently eclosed (<2-d-old) flies were transferred to a prechilled 50 ml Falcon tube and dark adapted by wrapping the tubes in aluminum foil. The tubes were then immersed in liquid nitrogen for 1 min, before vortexing for ~1 min to separate the heads. Heads were collected and freeze dried by storing in 10 ml ace- tone at −20°C for 3–4 d. Between 100 and 200 heads per sample were manually collected under a stereo microscope after draining the ace- tone and drying on Whatman filter paper and stored again at −20°C before lipid extraction. In some cases, retinas were dissected from the freeze-dried heads using a blunt, flattened insect pin (Matsumoto et al., 1982). Lipid extraction and lipidomic analysis of all major lipids and bis- and tris-phosphorylated phosphoinositides was performed as previously described (Clark et al., 2011; Norton et al., 2011) using an AB Sciex 4000QTRAP mass spectrometer connected to a Shimadzu Prominence HPLC system (Shimadzu Scientific Instruments).

Results
To manipulate membrane phospholipid composition, flies were reared on diets based on yeast, sucrose, and agar. Yeast
Lipids contain saturated and monounsaturated fatty acids, but little or no PUFAs (Ejsing et al., 2009; Carvalho et al., 2012). PUFAs content of the diet can thus be simply controlled by addition of specific PUFAs to the basic yeast diet. Yeast-based diets also lack vitamin A and flies reared on yeast have an ~500-fold reduction in rhodopsin (Isono et al., 1988). However, with addition of β-carotene (as precursor for the chromophore, 3-OH retinal), rhodopsin levels were restored to ~50% of normal, but not further influenced by addition of PUFAs supplement to the diet (Fig. 1B). Flies reared on the basic yeast + β-carotene diet (referred to as YF, for yeast food) were overtly normal with no obvious defects in morphology of the retina or photoreceptors (Fig. 1C). Nevertheless, in whole-cell recordings, despite overlap in values, there was on average a slight (~20%) reduction in capacitance (a sensitive measure of microvillar membrane area) in flies reared on both YF and LNA diets (Fig. 1D). The reduction in rhodopsin levels can be expected to reduce sensitivity (quantum catch) proportionally, but neither this, nor the slight reduction in microvillar area, would be expected to influence voltage-clamped response kinetics, which show no correlation with capacitance or rhodopsin level (R. C. Hardie, unpublished results). Importantly, YF-reared flies were indistinguishable in both respects from LNA-reared flies, which represent the most direct relevant control in most experiments.

**Lipidomic analysis**

The major classes of phospholipids, i.e., phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphoinositides (PI and PIPn), were extracted, separated, and analyzed by mass spectrometry. Lipid species were identified by total carbon number and number of unsaturated bonds in the acyl tails, e.g., 16:0, 18:1 (oleic acid) acyl tails or 18:2 (linoleic acid) plus 18:0 (stearic acid). According to previous lipidomic analysis (Ejsing et al., 2009; Carvalho et al., 2012), was also found for PC, PS, PI, and PIP (data not shown). D, Comparison of FA species in phospholipids in present study (heads from flies reared on normal diet) with a recent lipidomic study of purified rhabdomeric membranes (Munoz et al., 2013). For this comparison, our data (for both acyl species combined) have been assigned the most likely single acyl tails on the basis of published data from Drosophila (Carvalho et al., 2012) and summed across all phospholipid classes.
species such as 36:2 and 34:2 in yeast correspond to phospholipids with two monounsaturated acyl tails (e.g., 18:1 and/or 16:1), and PUFAs are present in only trace amounts across all lipid species in yeast. Our analysis confirmed this for the phospholipid species initially present in the yeast food, with an overall reduction of ~20-fold in the concentration of polyunsaturated phospholipids (with three or more double bonds in both tails considered together) compared with the plant-based control diet (1.8 µg/g in YF, cf. 37 µg/g in control food).

Lipidomic analysis of fly heads reared on the respective diets showed that the lack of PUFAs in the YF diet was reflected in corresponding marked differences in the fatty acid composition of all the major phospholipids in the head (Figs. 2, 3). In particular, while monounsaturated species were little affected, or increased in flies reared on the YF diet, unambiguous polyunsaturated species (with three or more double bonds: 36:3, 36:4) were greatly reduced in all classes of phospholipids (Figs. 2, 3). When reared on the normal diet, 38% of the total phospholipid pool contained at least one polyunsaturated acyl tail; however, on the YF diet this was reduced sevenfold to ~5%. Supplementation of the diet with a single PUFA species (LNA, 18:3 or LA, 18:2) effectively restored the proportion of polyunsaturated phospholipids, while saturated (PAL or ST) or a monounsaturated fatty acid (FA; OL, 18:1) failed to do so (Fig. 3B). Flies reared on the YF diet also tended to have slightly shorter acyl tails as previously reported (Carvalho et al., 2012). However, differences were very slight; for example, the average (combined) acyl tail length for PE (the major phospholipid in Drosophila) was 35.4 C atoms on control food, but not OL or PAL, rescued the short latency.

Figure 4. Light responses are slower in flies reared on PUFA-deficient diets. **A**, Representative responses to 1 ms flashes (arrows) containing ~80 effective photons in wild-type photoreceptors reared on normal diet (control), YF diet, and the same diet supplemented with 50 µl/100 ml LNA, OL, ST, and PAL. **B**, Time to peak of flash responses (as in A) in flies reared on control (mean ± SEM; n = 12 cells) and YF diets and YF diets with different fatty acid supplements. Time to peak in flies reared on all PUFA-deficient diets (YF, n = 9; ST, n = 5; PAL, n = 3; OL, n = 3 cells) were 2–3× longer than in control (p < 0.05, one-way ANOVA, Bonferroni correction). PUFAs (LNA, n = 8 and n = 13 cells) rescued the rapid response. **C**, Amplitude of responses tended to be reduced on PUFA-deficient diets but did not reach significance. **D**, Representative superimposed quantum bumps induced by brief (1 ms) flashes containing on average less than one effectively absorbed photon. Bumps had relatively short latencies in flies reared on normal diet but long and variable latencies on YF diets. LNA, but not OL or PAL, rescued the short latency. **E**, Averaged bump waveforms on control YF and LNA diets were indistinguishable. **F**, Representative examples of normalized bump latency distributions from photoreceptors of flies reared on control, YF, and LNA diets. **G**, Mean bump latency (~SEM across cells) on different diets were slower on YF, OL, ST, and PAL diets (p < 0.05; one-way ANOVA, Bonferroni correction). **H**, Mean bump amplitudes on different diets were similar.

This we dissected ~100 retinae from freeze-dried tissue of flies reared on each of the three main diets (normal, YF, and YF + LNA), and found closely overlapping profiles, with again a massive reduction in PUFAs (mean: 9.8-fold reduction aver-
Effect of diet on the light response

In whole-cell recordings made from dissociated ommatidia of flies reared on the YF diet, it was immediately apparent that responses to brief light flashes were greatly slowed (Fig. 4). As previously reported, time to peak in flies reared on normal food is typically ~40–60 ms, but in flies reared on YF diet responses were two to three times slower (~120 ms). Time to peak was restored to normal by addition of a single PUFA species (50 μl/100 ml) to the diet, with both LNA (18:3) and LA (18:2) rescuing the normal rapid response. In contrast, addition of the same amount of saturated (PAL and ST) or monounsaturated fatty acid (OL) to the diet resulted in no improvement (Fig. 4A, B).

The macroscopic response to a brief flash is that the linear summation of the underlying responses to single photons (quantum bumps) and kinetics is consequently jointly determined by the convolution of bump waveform and bump latency (Henderson et al., 2000). To see which was responsible for the slower responses, we recorded responses to single photons (quantum bumps) by delivering brief (~1 ms) dim flashes containing on average less than a single effective photon. Quantum bump amplitude (~9 pA), waveform, and duration (τ1/2 ~ 20 ms) were indistinguishable across all diets (Fig. 4E, H); however, bump latency was specifically prolonged and the latency distribution broadened on YF diet, once again fully rescued by addition of LNA or LA, but not saturated or monounsaturated fatty acids to the diet (Fig. 4D, F, G). In contrast, overall sensitivity (response amplitude) was little affected by diet. Because of the clear rescue of response kinetics by polyunsaturated, but not saturated or monounsaturated FA supplements, all subsequent experiments were performed on just three basic diets: normal (control) food, YF, and YF + LNA (LNA diet).

We next explored the consequence of dietary manipulation for visual performance in intact animals. First, recordings of voltage responses of photoreceptors using intracellular sharp microelectrodes in the intact eye confirmed that temporal resolution was compromised under physiological conditions in vivo. The latency of responses to brief light flashes was increased ~2-fold in YF-reared flies, and information rates assessed by noise analysis of responses to naturalistic image contrast modulation were significantly impaired due to a specific reduction in the SNR at frequencies above ~30 Hz (Fig. 5A–D). Second, we used a torque meter to record optomotor behavioral responses of intact flies in tethered flight. While yaw torque responses to aged across all phospholipid species) on the yeast diet (Fig. 3C). Furthermore we found that our fatty acid profile of fly heads reared on normal food very closely matched that recently reported for a fraction enriched in rhabdomeric membrane (Fig. 3D; Muñoz et al., 2013).
slowly moving stimuli (45° s⁻¹) were not significantly affected, responses to a fast-moving stimulus (180° s⁻¹) were severely suppressed (~4-fold) in flies reared on YF food (Fig. 5E,F). Both photoreceptor responses and optomotor behavior were restored to control levels by adding LNA to the yeast diet.

Quantum bumps are shaped by a sequence of Ca²⁺-dependent positive and negative feedback, which is believed to activate and inactivate the majority of channels in a single microvillus (Henderson et al., 2000). The positive feedback by Ca²⁺, which is highly nonlinear, could potentially compensate for and mask a more significant reduction in sensitivity of the channels to the primary activator. To explore sensitivity in the absence of Ca²⁺-dependent feedback, we recorded in Ca²⁺-free bath solution and found that sensitivity (peak amplitude to a standard intensity test flash) was now reduced ~10-fold on YF diet (Fig. 6A,B), but rescued on LNA diet. Time to peak in Ca²⁺-free bath was only slightly longer in flies reared on YF diet, presumably because under these conditions, the kinetics of the macroscopic response is now dominated by quantum bump lifetime (~200 ms in the absence of Ca²⁺ influx) rather than bump latency (Henderson et al., 2000). In addition, we recorded from trp mutant flies, expressing only TRPL channels, which unlike TRP channels are not subject to Ca²⁺-dependent positive feedback (Reuss et al., 1997). Time to peak was once again ~3× longer in trp flies reared on YF diet, and peak amplitude was now also greatly reduced (~7-fold), with both rescued as usual by LNA added to the diet (Fig. 6C,D).

Under normal conditions, recordings in the dark are characterized by "dark noise," an ongoing barrage of miniature (~2 pA) quantum bump-like events occurring at rates of ~2–4 s⁻¹, caused by spontaneous activation of Gq-proteins (Hardie et al., 2002; Elia et al., 2005; Chu et al., 2013b). In fact spontaneous G-protein activations are believed to be ~10X more frequent than even this, but it seems that most single activated Gq/PLC complexes fail to overcome the threshold for channel activation (Katz and Minke, 2012; Chu et al., 2013b). If increasing the degree of saturation of membrane phospholipids acts either by reducing PLC activity or its downstream actions, we predicted that dark noise would be suppressed by the YF diet. Indeed, in flies reared on YF diet spontaneous dark events were greatly reduced in

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Effect of diet on light responses without Ca²⁺-dependent positive feedback. A. Responses to 1 ms flashes (arrows) containing ~75 effective photons in whole-cell, patch-clamped wild-type photoreceptors in the presence and absence of Ca²⁺. The presence of Ca²⁺ responses in flies reared on YF diet were markedly reduced in amplitude (p < 0.0001), but rescued by LNA supplement to diet. B. Averaged peak response corrected for photon content (top) and t-pk (below) of responses in 0 Ca²⁺ bath (mean ± SEM; n = 8–10 cells). C. Responses to 1 ms flashes (arrows) containing ~75 effective photons in trp-mutant photoreceptors. Responses in flies reared on YF diet were slowed and reduced in amplitude (p < 0.005). D. Peak response (top) was ~7-fold reduced (p < 0.005) and t-pk (below) ~3-fold slower (p < 0.0001) in YF diet, but rescued by LNA supplement (mean ± SEM; n = 8–10 cells).
both frequency and amplitude, but restored by addition of LNA to the diet (Fig. 7A, B).

We also made recordings from hypomorphic $Gaq^1$ mutants where levels of $G_{q}$-protein $\alpha$-subunit are reduced to $\sim$1%, such that each microvillus only contains one or two G-proteins at most (Scott et al., 1995). $Gaq^1$ mutants are $\sim$1000-fold less sensitive than wild-type, due partly to smaller quantum bumps, but primarily reflecting a massive reduction in quantum efficiency. This is probably because each activated rhodopsin can, in most cases, activate only a single G-protein, and again most single G-protein/PLC complexes fail to overcome the threshold for channel activation (Scott et al., 1995; Hardie et al., 2002). Nevertheless, $Gaq^q$ mutants reared on normal food or LNA diet still generate robust responses of up to $\sim$1 nA with sufficiently bright stimuli (Fig. 7C, D). Remarkably, rearing $Gaq^q$ flies on YF diet almost completely eliminated any response to light at all, with 6/12 cells showing no detectable response to the brightest flashes tested ($\sim$10$^{-3}$ effective photons). The remainder gave tiny responses (<5 pA), consisting of a few noisy channel openings (Fig. 7C). To confirm this profound (>4 orders of magnitude) loss of sensitivity in vivo, we also made electroretinogram (ERG) recordings from intact flies. $Gaq^q$ mutants reared on normal food or LNA diet generated responses of up to 5–10 mV over an $\sim$4 log unit dynamic range. However, in flies reared on YF diet, the response was almost entirely abolished, with at most, a tiny response of $\sim$0.2 mV detected by averaging repeated responses to the brightest intensity (Fig. 7E, F).

**Na$^+$/Ca$^{2+}$ exchange activity is not affected by diet**

The threshold for quantum bump generation is strongly dependent on the Ca$^{2+}$ concentration in the microvilli (Henderson et al., 2000; Katz and Minke, 2012; Chu et al., 2013b), which in turn is largely controlled by the activity of a Na$^+$/Ca$^{2+}$ exchanger encoded by the $\text{calX}$ gene (Wang et al., 2005). We therefore asked whether the suppressed sensitivity on the YF diet might be due to a reduction in [Ca$^{2+}$] mediated by an increase in Na$^+/Ca^{2+}$ exchanger activity, which can be modulated by lipids such as PIP$_2$ (Hilgemann and Ball, 1996). The Na$^+/Ca^{2+}$ exchanger is electrogenic and its activity can be directly measured as an aftercurrent in response to bright light flashes (Chu et al., 2013a; Fig. 8A, B). In wild-type photoreceptors reared on normal food, this reaches a maximum of $\sim$150 pA decaying with a characteristic time constant of $\sim$300 ms as the Ca$^{2+}$, which entered during the light-induced TRP and TRPL current is extruded (Chu et al., 2013a). Both the amplitude and decay time constant of the Na$^+/Ca^{2+}$ aftercurrent were similar in flies reared on YF diet and yeast with LNA supplement, indicating that there was no significant effect of diet on exchanger activity (Fig. 8C).
PLC activity is little altered by diet

Other possible explanations for the effects of the YF diet include a reduction in the rate of PIP₂ hydrolysis by PLC, or a reduced ability of the products or consequences of PIP₂ hydrolysis to gate the channels. To distinguish these possibilities, we asked whether the rate of PIP₂ hydrolysis by PLC was influenced by diet. To monitor PLC activity in real time we measured the pH change resulting from the PLC reaction, which releases a proton for each PIP₂ hydrolyzed. As previously reported, light induces a rapid PLC-dependent acidification that can be measured with a fluorescent pH indicator (HPTS) loaded into the photoreceptor via the patch pipette (Huang et al., 2010). In wild-type flies, the blue excitation light used to measure HPTS fluorescence is also simultaneously a supersaturating stimulus for the cell (equivalent to ~10⁴ effectively absorbed photons per second). To measure pH changes in response to less saturating illumination, we recorded HPTS fluorescence in photoreceptors from flies in which the blue-absorbing Rh1 rhodopsin had been replaced by a UV opsin (Rh3) absorbing maximally at ~340 nm (Feiler et al., 1992; Wardill et al., 2012). In these flies, the blue excitation light now elicited only a small light-induced current, and any pH shift was below the limits of detection. UV light (390 nm) superimposed on the blue light evoked larger electrical responses and resulted in rapid reduction in HPTS fluorescence indicating acidification (Fig. 9). At these intensities (equivalent to ~10⁵–10⁶ effectively absorbed photons per second), the absolute rates of initial acidification between flies reared on YF or LNA were indistinguishable and there was no significant difference between any of the diets. In fact the overall pH shift was ~2× greater in YF-reared flies, but this was because pH continued to fall for about twice as long in flies reared on YF than on YF-reared YF diet (Fig. 9C). Previously we reported that PLC activity is rapidly inhibited by Ca²⁺ influx via PKC (Gu et al., 2005; Huang et al., 2010); hence this difference can be readily attributed to the slower onset of Ca²⁺-dependent inhibition of PLC due to the longer response latency in flies reared on the YF diet (Fig. 9D). Overall these results suggest that there is little if any direct effect of diet and phospholipid composition upon PLC activity.

Effect of diet on photomechanical responses

The results thus far indicate that the degree of saturation of membrane phospholipids influences the gain and kinetics of the light response downstream of PLC. Because increasing the amount of saturated phospholipids in a membrane increases its stiffness and decreases its flexibility (Brenner, 1984; Rawicz et al., 2000), this would be consistent with the hypothesis that the channels are gated mechanically, in combination with protons, as recently proposed (Hardie and Franze, 2012). We used two approaches to test whether dietary manipulation had indeed affected the photomechanical response of the photoreceptors.

First, as a “molecular force transducer,” we incorporated the mechanosensitive ion channel, gramicidin (Andersen et al., 1999; Andersen and Koepe, 2007), into membranes of photoreceptors in which native light-sensitive channel activity had been eliminated by a combination of mutation (trpCl) and pharmacological block (100 μM La³⁺ and 20 μM ruthenium red). Photoreceptors were loaded with gramicidin (0.05–0.1 ng/ml) via puffer pipette perfusion resulting in the gradual development of a constitutive inward current mediated by gramicidin channels (Fig. 10A). Before the addition of gramicidin, bright test flashes under these conditions only elicited a tiny biphasic current (Fig. 10B), which may represent the electrical signature of the charge displacement as negatively charged InsP₃ is released from the membrane by PLC (Huang et al., 2010). As previously reported (Hardie and Franze, 2012), after incorporation of gramicidin channels into the membrane, the same test flash (~10⁷ effective photons) elicited, in addition, a rapid 15–20% increase in the gramicidin-mediated inward current, which we attribute to the change in the physical properties of the bilayer upon PIP₂ hydrolysis. Light-elicited gramicidin responses in flies reared on normal diet and LNA diets were indistinguishable and could be well fitted by a single exponential with a time constant of ~250 ms (Fig. 10B–D). However, in flies reared on YF diet, the responses were ~2-fold slower (tau ~500 ms). Responses on YF diet also tended to be slightly larger in amplitude, but this did not reach statistical significance (p = 0.12, one-way ANOVA).

In a second approach, we also directly measured light-induced contractions of retinas in flies reared on different diets using atomic force microscopy (Franze, 2011; Hardie and Franze,
Experiments were again performed on trpl mutants expressing only TRP channels, because it is then possible to completely block all light-sensitive channel activity by perfusion with La$^{3+}$ (see below). As previously described, brief light flashes evoked rapid contractions of the photoreceptors, which we interpret as a narrowing of the microvillar diameters when PIP$_2$ is hydrolyzed from the inner leaflet of the lipid bilayer (Hardie and Franze, 2012). The maximum contractions evoked in flies reared on YF diet tended to be slightly larger than on normal or LNA diets, but also peaked later (time to peak 172 ± 31 ms, cf. 131 ± 10.5 ms on control food and 119 ± 25.6 ms on LNA diet). This can again be interpreted in terms of the delayed onset in the Ca$^{2+}$ influx required to terminate PLC activity in flies reared on YF diet. Significantly however, and in contrast to PLC activity itself (Fig. 9), the contractions elicited from retinas of flies reared on YF diet were consistently slower than in flies reared on normal food or yeast with LNA supplement (Fig. 11). To avoid feedback effects due to Ca$^{2+}$ influx, we also made measurements from trpl retinas after perfusion with La$^{3+}$ (100 μM) and ruthenium red (10 μM) to block all light-sensitive TRP channel activity. As previously reported (Hardie and Franze, 2012), the magnitude of the contractions was enhanced (because of the now total lack of Ca$^{2+}$-dependent inhibition of PLC), but once again contractions recorded in Ca$^{2+}$-dependent positive feedback acting on TRP channels. In the absence of Ca$^{2+}$ influx or in trp mutants expressing only TRPL channels, sensitivity was reduced up to ~10-fold, and essentially abolished in hypomorphic Gaq$^+$ mutants. Our results indicate that these effects, all of which were rescued by the addition of a single species of PUFA to the yeast diet, were mediated predominantly downstream of PLC and were associated with slowing of photomechanical responses.

**Discussion**

The ability to manipulate fatty acid composition of phospholipids in flies by diet has been reported previously (Zinkler et al., 1985; Stark et al., 1993; Shen et al., 2010; Carvalho et al., 2012). However, to our knowledge, there are no convincing reports of physiological consequences. Here, we found that the proportion of polyunsaturated phospholipids in flies reared on yeast was reduced ~7-fold (Fig. 3), and that this was associated with pronounced effects on visual performance, with photoreceptor responses slowed 2- to 3-fold, without significantly affecting quantum bump waveform, Na$^+$/Ca$^{2+}$ exchange, or PLC activity. Although absolute sensitivity was little affected by diet in wild-type photoreceptors, this could be attributed to compensation by light-induced calcium influx or in

**Mechanism of activation**

The macroscopic light response in *Drosophila* reflects the summation of quantum bumps, each arising from activation of most of the ~20 TRP channels in a single microvillus. Briefly, a single activated rhodopsin is believed to activate ~5 or so G$_q$-proteins by random diffusional encounters, and each released G$_q$ α-subunit diffuses further before binding and activating PLC. Each PLC molecule rapidly hydrolyzes PIP$_2$, building up sufficient excitatory “messenger” to overcome a finite threshold required to activate the first TRP channel with a stochastically variable latency of ~15–100 ms. The resulting Ca$^{2+}$ influx raises Ca$^{2+}$ throughout the microvillus into the micromolar range within milliseconds, facilitating activation of the remaining channels, and generating an “all-or-none” quantum bump local-
ized to a single microvillus. This raises Ca\(^{2+}\) within the affected microvillus to \(~1\, \text{mm}\) terminating the bump by Ca\(^{2+}\)-dependent inactivation of the channels and preceding steps of the cascade (for reviews and computational models see Postma et al., 1999; Pumir et al., 2008; Hardie, 2012; Song et al., 2012). This highly nonlinear positive and negative feedback cycle, which shapes the quantum bump waveform, was apparently not influenced by diet (Fig. 4 E, H). However, bump latency, i.e., the time taken to activate the first channel, was clearly profoundly delayed in flies reared on the YF diet (Fig. 4 D, F, G).

Which products of PLC activity are responsible for this initial gating remains controversial. InsP\(_3\) and Ca\(^{2+}\) stores apparently play no role, because mutants of the InsP\(_3\) receptor have normal phototransduction (Acharya et al., 1997; Raghu et al., 2000). Alternative candidates include DAG or PUFAs, which might be released from DAG by an appropriate lipase. Of these, DAG has generally proved ineffective as an agonist when exogenously applied; however, there is genetic evidence, based on mutants of DAG kinase (rdgA), for DAG as an excitatory messenger (for review, see Raghu and Hardie, 2009; Hardie, 2012). One lab has also reported that DAG can activate TRP channels in excised patches from dissociated rhabdomeres (Delgado and Bacigalupo, 2009; Delgado et al., 2014); however, activation was very sluggish with delays of up to 60 s, in a preparation that is in a physiologically severely compromised state.

In contrast, there is universal consensus that PUFAs are very effective agonists when exogenously applied to both native channels in the photoreceptors and heterologously expressed TRPL channels. Apparent support for endogenous PUFAs came from the reduced light response found in a Drosophila DAG lipase mutant, inaE (Leung et al., 2008). However, inaE encodes an \(sn-1\) DAG lipase, which rather than PUFAs, releases mono-acyl glycerols (MAGs), which are at best weak and slowly acting channel agonists when applied exogenously (Hardie, unpublished results). For PUFAs generation, either an \(sn-2\) DAG lipase or an additional enzyme (MAG lipase) would be required, but there is no evidence for either in photoreceptors. Furthermore, the inaE gene product immunolocalizes to the cell body with, at most, occasional puncta in the rhabdomere (Leung et al., 2008), and there is no evidence that PUFAs are generated in response to illumination (Delgado et al., 2014).

An alternative hypothesis comes from evidence showing that the channels can be activated by the strict combination of two further consequences of PLC action, namely PIP, depletion and proton release (Huang et al., 2010). Furthermore it was suggested that PIP\(_2\)'s role is mediated not by ligand binding/unbinding but by the physical effects of PIP\(_2\) depletion on the lipid bilayer (Hardie and Franze, 2012). Thus, removal of PIP\(_2\)'s inositol headgroup from the inner leaflet effectively reduces membrane area, and it was proposed that the resulting mechanical effects (on, e.g., membrane tension, curvature, or lateral pressure) lead to mechanical gating of the channels, in combination with protons. Evidence supporting this included: (1) the ability of light to activate ectopic mechanosensitive channels (gramicidin); (2) facilitation of light responses by hypotonic solutions; and (3) rapid contractions of the photoreceptors in response to light, interpreted as the concerted contraction of microvilli due to PIP\(_2\) hydrolysis in the inner leaflet (Hardie and Franze, 2012).

In the present study we used diet to increase the degree of saturation of the phospholipids, which should make membranes...
stiffer and less flexible (Brenner, 1984; Rawicz et al., 2000), and we predicted that this might suppress mechanosensitive channel activity. Lipidomic analysis confirmed a drastic sevenfold reduction in PUFA content across all phospholipid species in YF-reared flies. This was associated with a 2- to 3-fold longer latency and a marked reduction in open probability once Ca\(^{2+}\)-dependent positive feedback was eliminated (Figs. 4, 6). In principle PLC activity might be suppressed by the YF diet, because

**Figure 11.** AFM measurements of light-induced contractions. **A,** AFM responses (contractions in nanometers) to 2 ms flashes of increasing intensity (\(\sim 10^3 \text{--} 10^5 \) effective photons, delivered at \(t = 0\)) in retinas dissected from trpl mutant flies: left, reared on YF diet (traces averaged from \(n = 8\) retinas); right, on LNA (blue traces, \(n = 9\) ) and control diets (black traces, \(n = 9\). **B,** Comparison of contractions elicited at three different intensities (\(10^3\), \(4 \times 10^4\), and \(2 \times 10^5\) effective photons) on the three diets (traces i–iii from A on faster time bases). In all cases retinas from flies reared on YF diet gave slower contractions. **C,** Responses to the same intensity flashes after complete block of light-sensitive channels with La\(^{3+}\) (100 \(\mu\)M) and ruthenium red (RR; 10 \(\mu\)M). The contractions in YF flies (\(n = 10\) ) were again slower than control (\(n = 5\) ) and LNA-reared flies (\(n = 7\) ). **D,** Response intensity function (mean \(\pm\) SEM. of maximum contractions plotted against effective intensity). **E,** Latency to first detectable contraction as a function of intensity in flies reared on different diets in normal bath solution. **F,** Latency measured from contractions after channel block by La\(^{3+}\) and RR.
membrane fluidity, and hence diffusion (e.g., of G-proteins, or PIP$_2$), might be slower in a membrane dominated by saturated phospholipids. However, molecular dynamic simulations predict that lateral diffusion coefficients in fact depend only weakly on the degree of phospholipid saturation (Ollila et al., 2007). Importantly, the rate of PIP$_2$ hydrolysis monitored by proton release appeared unaffected by diet (Fig. 9) suggesting that diet was acting primarily downstream of PLC. That dietary manipulation had indeed resulted in alteration to the mechanical properties of the membrane was supported by AFM measurements of the light-induced contractions and responses mediated by ectopic mechanosensitive gramicidin channels. These indicated that, despite the lack of effect on PLC activity, YF-reared flies generated slower photomechanical responses.

Although consistent with the mechanical gating hypothesis, by themselves our data do not exclude the alternative suggestions, that the excitatory messenger is DAG or a PUFA. As suggested by recent studies of mechanosensation (Vázquez et al., 2014) and rapid endocytosis (Pinot et al., 2014), underlying mechanisms are poorly understood, and in this respect, our results provide a striking and novel example of how dietary fatty acids can profoundly and specifically influence in vivo performance and behavior via a defined step within the context of a classical G-protein-coupled signaling cascade. Interestingly, the importance of the mechanical properties of membranes containing polyunsaturated phospholipids has also been highlighted in recent studies of mechanoassays (Vázquez et al., 2014) and rapid endocytosis (Pinot et al., 2014).

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