Phospholipase C-Mediated Suppression of Dark Noise Enables Single-Photon Detection in *Drosophila* Photoreceptors

Ben Katz and Baruch Minke

Departments of Medical Neurobiology, the Institute of Medical Research Israel-Canada, the Edmond and Lily Safra Center for Brain Sciences, and the Kühne Minerva Center for Studies of Visual Transduction, Faculty of Medicine of the Hebrew University, Jerusalem 91120, Israel

Drosophila photoreceptor cells use the ubiquitous G-protein-mediated phospholipase C (PLC) cascade to achieve ultimate single-photon sensitivity. This is manifested in the single-photon responses (quantum bumps). In photoreceptor cells, dark activation of $G_q\alpha$ molecules occurs spontaneously and produces unitary dark events (dark bumps). A high rate of spontaneous $G_q\alpha$ activation and dark bump production potentially hampers single-photon detection. We found that in wild-type flies the *in vivo* rate of spontaneous $G_q\alpha$ activation is very high. Nevertheless, this high rate is not manifested in a substantially high rate of dark bumps. Therefore, it is unclear how phototransduction suppresses dark bump production arising from spontaneous $G_q\alpha$ activation, while still maintaining high-fidelity representation of single photons. In this study we show that reduced PLC catalytic activity selectively suppressed production of dark bumps but not light-induced bumps. Manipulations of PLC activity using PLC mutant flies and Ca^{2+} modulations revealed that a critical level of PLC activity is required to induce bump production. The required minimal level of PLC activity selectively suppressed random production of single $G_q\alpha$ -activated dark bumps despite a high rate of spontaneous $G_q\alpha$ activation. This minimal PLC activity level is reliably obtained by photon-induced synchronized activation of several neighboring $G_q\alpha$ molecules activating several PLC molecules, but not by random activation of single $G_q\alpha$ molecules. We thus demonstrate how a G-protein-mediated transduction system, with PLC as its target, selectively suppresses its intrinsic noise while preserving reliable signaling.

Introduction

Fly photoreceptors use G-protein-mediated phospholipase C (PLC) signaling to achieve ultimate sensitivity to single photons, as manifested in single-photon responses (quantum bumps; Yem and Spiegel, 1973; Wu and Pak, 1975). Reliable single-photon detection requires accurate differentiation between quantum bumps and dark noise, which in *Drosophila*, mainly arises from unitary events that are similar in shape to quantum bumps but smaller in amplitude (dark bumps; Hardie et al., 2002; Elia et al., 2005). The dark bumps are thought to originate from spontaneous $G_q\alpha$ activation, as evidenced by absence of dark bumps in the $G_q\alpha^1$ mutant (Hardie et al., 2002) and by the re-

duced spontaneous G-protein activation when $G_q\beta$ is found in excess over $G_q\alpha$ (Elia et al., 2005).

PLC is a key enzyme in fly phototransduction with transient receptor potential (TRP) channels as its targets (Devary et al., 1987; Bloomquist et al., 1988; Selinger and Minke, 1988). In *Drosophila* photoreceptors the *norpA* (*no receptor potential A*) gene encodes a β -class PLC, predominately expressed in the signaling compartment (the rhabdomere). Mutations in the *norpA* gene causing reduced levels of the protein show reduced receptor potential amplitude and slow response termination (Bloomquist et al., 1988). Thus, although PLC β is a functional phospholipase, the latter phenotype has led to the discovery that PLC functions as a GTPase-activating protein (GAP) as well. This apparent inability to hydrolyze GTP bound to $G_q\alpha$ without PLC ensures that every activated G-protein eventually encounters a PLC molecule required for TRP channel activation (Cook et al., 2000). However, the mechanism underlying TRP and TRPL channel gating, downstream of PLC activation, remains unresolved (but see Chyb et al., 1999; Leung et al., 2008; Delgado and Bacigalupo, 2009; Katz and Minke, 2009; Parnas et al., 2009; Huang et al., 2010).

Ca^{2+} plays a major role in excitation, positive and negative feedback regulation (Hardie, 1991), and adaptation (Gu et al., 2005) of the *Drosophila* response to light. These processes are attributed to the regulatory effects of Ca^{2+} on many phototransduction proteins including PLC (Toyoshima et al., 1990; Run-

Received Oct. 17, 2011; revised Dec. 22, 2011; accepted Dec. 30, 2011.

Author contributions: B.K. and B.M. designed research; B.K. performed research; B.K. analyzed data; B.K. and B.M. wrote the paper.

This research was supported by grants from the National Eye Institute (R01 EY 03529), the US-Israel Binational Science Foundation, the Israel Science Foundation, and the German Israeli Foundation. We thank Drs. François Payre and Armin Huber for the antibodies against Dmoesin and PLC, respectively; Dr. William L. Pak for the PLC mutants; and Drs. Roger Hardie, Alain Pumir, Johannes Oberwinkler, and Boaz Cook for valuable discussions, for useful comments, and for critical reading of the manuscript. We also thank Drs. Moshe Parnas, Shahar Frechter, Shaya Lev, Maximilian Peters, and David Zeevi for critical reading of the manuscript.

Correspondence should be addressed to Baruch Minke, Department of Medical Neurobiology, Faculty of Medicine, The Hebrew University, P.O. Box 12272, Jerusalem 91120, Israel. E-mail: baruchm@ekmd.huji.ac.il.

DOI:10.1523/JNEUROSCI.5221-11.2012

Copyright © 2012 the authors 0270-6474/12/322722-12\$15.00/0

Table 1. Extracellular solution

Solution name	NaCl (mM)	KCl (mM)	MgSO ₄ (mM)	TES (mM)	MgCl ₂ (mM)	CaCl ₂ (mM)	SrCl ₂ (mM)	BaCl ₂ (mM)	L-Proline (mM)	L-Alanine (mM)
Low Ca ²⁺	125	5	—	10	—	—	—	—	25	5
1.5 mM Mg ²⁺	125	5	—	10	1.5	—	—	—	25	5
1.5 mM Ca ²⁺	125	5	—	10	—	1.5	—	—	25	5
1.5 mM Sr ²⁺	125	5	—	10	—	—	1.5	—	25	5
1.5 mM Ba ²⁺	125	5	—	10	—	—	—	1.5	25	5
1.5 mM Ca ²⁺ + 4 mM Mg ²⁺	120	5	4	10	—	1.5	—	—	25	5

ning Deer et al., 1995; Hardie, 2005). Ca²⁺ has profound effects on response kinetics (Hardie and Raghu, 2001; Katz and Minke, 2009), but the target proteins and mechanisms are not entirely clear. Recent studies have presented quantitative models that explain how bumps emerge from stochastic nonlinear dynamics of phototransduction. These models explain the reliability of bump formation, effects of external Ca²⁺ concentration ([Ca²⁺]) on bump kinetics, and the low background noise in the dark (Pumir et al., 2008; Nikolic et al., 2010). However, the detailed molecular mechanisms differ in the two models, especially with regard to the mechanism underlying dark noise suppression.

In the present study we investigated the mechanism that suppresses dark bump production yet still maintains high-fidelity representation of single photons. This suppression, which occurs despite a relatively high rate of spontaneous G_qα activation, results from the requirement of a minimal level of PLC activity that is necessary to trigger bump production. The required level of PLC activity is rarely obtained by random activation of single G_qα molecules but always obtained by photon-induced synchronized activation of several neighboring G_qα molecules that activate several PLC molecules.

Materials and Methods

Fly stocks. Flies (*Drosophila melanogaster*) of either sex were raised at 24°C in a 12 h light/dark cycle on standard medium. Pupae vials were wrapped with aluminum foil and moved into a dark box 12 h before eclosion (dark adapted) or placed 10 cm from white fluorescent light (L 36, 20 W; OSRAM) 4–6 h before eclosion (light adapted).

Western blot analysis. For detection of NORPA and G_qα proteins, 5 newly eclosed dark raised fly heads were used for each lane of Western blots. Proteins were extracted with 1× SDS-PAGE buffer (2% SDS, 100 mM DTT, 10% glycerol in 65 mM Tris-HCl, pH 6.8) and subjected to 6% or 10% SDS-polyacrylamide gels for norpA or G_qα, respectively (Midget System, GE Healthcare). Protein levels were detected using anti-G_q (1:2000), anti-PLC (1:500) and anti-Dmoein antibodies (1:10,000). Relative protein amounts on the same gel were determined by quantification of the ECL signal on an exposed film. Bands on the exposed film were quantified using TINA2.0 software. To reduce the variance caused by the experimental procedure, the density in each lane was corrected by the αMoesin signal in the same lane and calculated as a percentage of WT fly signals.

Light stimulation. A xenon high-pressure lamp (PTI, LPS 220, operating at 75 W) was used and the light stimuli were delivered to the ommatidia by means of epi-illumination via the objective lens (*in situ*). The intensity of the orange light (Schott OG 590 edge filter) at the specimen was calibrated by measuring bump rate from WT flies at low Ca²⁺ conditions (effective photons per second).

Solutions. Components of extracellular and intracellular solutions are listed in Tables 1 and 2, respectively. All solutions were titrated to pH 7.15.

Electrophysiology. Dissociated *Drosophila* ommatidia were prepared as previously described (Peretz et al., 1994) from newly eclosed flies (<1 h after eclosion) and transferred to a recording chamber on an inverted Olympus microscope. Whole-cell recording were performed at 21°C using borosilicate patch pipettes of 8–12 MΩ, an Axopatch 1D (Molecular

Table 2. Intracellular solution

Solution name	D-Gluconic acid potassium salt (KGlu, mM)	MgSO ₄ (mM)	TES (mM)	MgATP (mM)	NaGTP (mM)	GDPTris (mM)	NAD (mM)	CaCl ₂ (mM)
KGlu (no GDP or '0' mM [Ca ²⁺] _i)	140	2	10	4	0.4	—	1	—
KGlu + 0.1 mM Ca ²⁺ (0.1 mM [Ca ²⁺] _i)	140	2	10	4	0.4	—	1	0.1
KGlu + 0.5 mM Ca ²⁺ (0.5 mM [Ca ²⁺] _i)	140	2	10	4	0.4	—	1	0.5
KGlu + 1 mM Ca ²⁺ (1 mM [Ca ²⁺] _i)	140	2	10	4	0.4	—	1	1
KGlu (–ATP)	140	6	10	—	0.4	—	1	—
KGlu (1 mM ATP)	140	5	10	1	0.4	—	1	—
KGlu (8 mM GDP)	132	2	10	4	0.4	8	1	—

Devices) voltage-clamp amplifier, Digidata 1440A and pClamp 10.2.0.14 software (Molecular Devices). Currents were filtered using the 8-pole low-pass Bessel filter of the patch-clamp amplifier at 5 kHz. Series resistance values were <25 MΩ and were routinely compensated to >80% when recording macroscopic responses >100 pA, but not when recording bumps.

Bump detection. Bumps were detected offline using the event detection threshold search function of pClamp 10.2.0.14 software (Molecular Devices). The following parameters were used: trigger, –3 pA; re-arm, –2 pA; pretrigger, 1 ms; post-trigger, 1 ms; and minimum allowed duration, 10 ms.

Results

Low Ca²⁺ selectively abolishes dark bumps but not quantum bumps

In *Drosophila* photoreceptor cells, dark bump production is thought to occur by spontaneous GDP-GTP exchange on the G_qα molecules. This notion leads to at least two crucial predictions: (1) dark bump rate should depend linearly on the amount of G_qα in the rhabdomeric membrane; (2) unfavorable conditions for GDP-GTP exchange should decrease the dark bump rate. To examine whether these predictions are realized, we measured the dark bump rate in flies with different expression levels of G_qα in the rhabdomeric membrane. To this end we used G-protein mutants and light vs dark rearing conditions which result in G_qα translocation. Accordingly, raising WT (*w¹¹¹⁸*) and mutant G_qα¹ heterozygote (*G_qα¹/+*) flies either in light or darkness changed G_qα distribution between the rhabdomeric membrane and the cytoplasm (Kosloff et al., 2003; Frechter et al., 2007). We found that the dark bump rate depended linearly on G_qα concentration bound to the rhabdomeric membrane, indicating that dark bump rate depends on activation of individual G_qα in the signaling compartment (Fig. 1A,B). Moreover, we used pipette solution with a high (8 mM) concentration of GDP (Table 2), which elevated cellular GDP concentration, thereby shifting the equilibrium toward G_qα-GDP. This manipulation largely decreased dark bump rate in WT flies (Fig. 1C,D). To further establish the robustness of dark bump suppression by

elevated cellular GDP, we performed the experiment in $G\beta e^1$ heterozygote ($G\beta e^1/+$). $G\beta e^1/+$ mutant show a high dark bump rate compared with WT flies in 1.5 mM Ca^{2+} containing solution ($\sim 7 s^{-1}$, $\sim 400\%$ of WT, Fig. 1C,D; Elia et al., 2005). Interestingly, elevation of cellular GDP also decreased dark bump rate in $G\beta e^1/+$ mutant flies (Fig. 1C,D). The above results indicated that the appearance of dark bumps arises from spontaneous GDP-GTP exchange, which is consistent with previous hypotheses (Hardie et al., 2002; Elia et al., 2005; Fig. 1).

The dark bumps have, on the average, a quarter smaller amplitude relative to quantum bumps in normal Ringer's solution containing 1.5 mM Ca^{2+} and 4 mM Mg^{2+} (Hardie et al., 2002). This relatively small amplitude of the dark bumps can be increased by removing external Mg^{2+} (Hardie and Mojette, 1995; Parnas et al., 2007). We therefore removed Mg^{2+} from the external solution throughout this study to reliably detect the occurrence of dark bumps.

When recording from WT photoreceptors in Ca^{2+} containing extracellular solution at total darkness, small-amplitude dark bumps are readily observed ($\sim 2 s^{-1}$, Fig. 2A,B). This low dark bump rate is not expected to hamper quantum bump detection as they rarely sum up. Strikingly, when Ca^{2+} was removed from the external solution (low Ca^{2+}), dark bump activity was eliminated (Fig. 2A–C). This phenomenon was not accompanied by the impairment of light excitation, as quantum bumps of high amplitude but of slower waveform were readily elicited under this condition (Fig. 2A; for measurements of bump kinetics, see Fig. 9). In general, when Ca^{2+} was omitted from the extracellular solution, dark bumps were virtually absent ($< 2 min^{-1}$) while, in the presence of Ca^{2+} , dark bumps were readily seen (Fig. 2B,C). To further establish the robustness of dark bump disappearance under low external Ca^{2+} , we explored the Ca^{2+} dependence of dark bumps in $G\beta e^1/+$ mutant flies. Removing extracellular Ca^{2+} virtually abolished the observed high rate of dark bump activity also in the $G\beta e^1/+$ mutant (Fig. 2B,C).

To further analyze the Ca^{2+} dependence of dark bump production, we elevated intracellular $[Ca^{2+}]_i$ in the recording pipette in a dose-dependent manner, while keeping low Ca^{2+} solution in the bath. Elevation of $[Ca^{2+}]_i$ up to 1 mM, significantly increased the formation of dark bumps in WT flies (Fig. 3, Table 2). The need for a very high Ca^{2+} concentration in the recording pipette to induce dark bumps is explained by the existence of a high concentration of intracellular Ca^{2+} buffers in addition to a powerful Ca^{2+} extrusion system, which slow down free diffusion of Ca^{2+} between the cell body and rhabdomere (Oberwinkler and Stavenga, 2000). Figure 3 suggests that a Ca^{2+} -dependent protein/s is specifically required for dark bump production and that Ca^{2+} is a limiting factor for dark bump production.

Figure 1. Dark bumps are the result of spontaneous GDP-GTP exchange on the $G_q\alpha$ subunit. **A**, Histogram plotting the mean bump rate of dark bumps of WT and $G_q\alpha^1$ heterozygote ($G_q\alpha^1/+$) and homozygote mutant raised either in the dark or in the light ($n = 5$, mean $\pm$ SEM). **B**, Scatter plot of dark bump rate as a function of $G_q\alpha$ protein levels in the membrane of different mutants and rearing conditions as in **A**. Note the highly significant linear relationship between these two parameters [mean $\pm$ SEM; data of $G_q\alpha$ protein levels in the membrane were taken from the work of Frechter et al. (2007)]. **C**, Whole-cell recordings of dark bumps from WT and $G\beta e^1$ heterozygote ($G\beta e^1/+$) mutant flies using extracellular solution with 1.5 mM Ca^{2+} and a pipette solution with either KGlu (no GDP) or KGlu (8 mM GDP) (Table 2). **D**, Histogram plotting the mean dark bump rate of the mutant flies at the designated conditions. Note that adding GDP to the recording pipette decreases dark bump rate ($n = 5$, mean $\pm$ SEM, $*p < 0.05$, $***p < 0.001$).

High rate of dark bumps hampers single-photon detection

The appearance of dark bumps is likely to hamper single-photon detection. To quantify the “dark noise” we plotted the root mean square (RMS) of current fluctuations recorded during dark and dim lights (Fig. 2A,B,D). The figure shows that elimination of the dark bumps under low Ca^{2+} conditions reduced the RMS [RMS; WT Dark (low Ca^{2+}) = 1.15, WT Dark (1.5 mM Ca^{2+}) = 2.1 Figs. 2A,D, 4F]. However, dark bumps appear at a low rate of $\sim 2 s^{-1}$ (Fig. 2C; Hardie et al., 2002). This relatively low rate of dark bumps might not impose a difficulty for reliably detecting the light-induced bumps at dim light, due to the smaller amplitude of the dark bumps relative to the quantum bumps (Fig. 2A). However, at higher dark bump rates of $> 8 s^{-1}$, bumps occasionally overlap, sum up and produce currents similar in size to the quantum bumps (Fig. 2B, arrows). Such high rates of dark bumps are observed in the $G\beta e^1/+$ mutant (Fig. 2B), which hampers single-photon detection. This is manifested in the RMS similarity of WT illuminated by dim light and the $G\beta e^1/+$ mutant in darkness (RMS; WT Light (1.5 mM Ca^{2+}) = 4.8, $G\beta e^1/+$ Dark (1.5 mM Ca^{2+}) = 3.8, Figs. 2D, 4F). This observation emphasizes the strong effect that dark bump rate has on dark noise (Fig. 2B,D; Elia et al., 2005).

The disappearance of dark bumps under low Ca^{2+} conditions in WT fly and $G\beta e^1/+$ mutant, may be explained by Ca^{2+} regulation of spontaneous GDP-GTP exchange on $G_q\alpha$, even though the activation of rhodopsin can facilitate exchange in the absence of Ca^{2+} . To test this possibility we examined whether spontaneous exchange continues under low Ca^{2+} conditions but is not manifested in bump production. To this end, we explored

Figure 2. Reduced external Ca^{2+} eliminates dark bump activity while the amplitudes of light-induced bumps are enhanced. **A**, Whole-cell recordings from WT flies in total darkness and in dim light stimulation of 1 effective photon/s (EP/s) under 1.5 mM Ca^{2+} (top trace) and low Ca^{2+} conditions (bottom trace). Note that no dark bump activity is seen under low Ca^{2+} conditions, while a response to light stimulation is observed. In extracellular solution containing 1.5 mM Ca^{2+} , dark bumps appear and light response is maintained. **B**, Whole-cell recordings from WT and *Gβe1/+* mutant flies at total darkness under 1.5 mM Ca^{2+} and low Ca^{2+} conditions. Note the summation of dark bumps (arrow). Also note the high dark bump rate of *Gβe1/+* mutant flies and the highly reduced dark bump activity under low Ca^{2+} conditions. **C**, Histogram plotting the mean dark bump rate of the mutant flies at the designated conditions ($n = 5$, mean $\pm$ SEM, ** $p < 0.01$, *** $p < 0.001$). **D**, RMS of current fluctuation of dark and light (1 EP/s) responses, at the designated conditions ($n = 5$, mean $\pm$ SEM). Note, the low RMS under low Ca^{2+} compared with 1.5 mM Ca^{2+} conditions in the dark and the high RMS at low Ca^{2+} compared with 1.5 mM Ca^{2+} conditions in the light. Also, note the similarity between the RMS of *Gβe1/+* dark and WT light under 1.5 mM Ca^{2+} . **E**, Whole-cell recordings in total darkness from *Gβe1/+* mutant flies under low Ca^{2+} conditions with intracellular solution without ATP (top trace) or with 1 mM ATP in the pipette (bottom trace) ($n = 3$). Note that dark bumps reappear at these conditions.

whether spontaneous exchange takes place under low Ca^{2+} conditions, when ATP was removed from the recording pipette (Fig. 2E, Table 2). It has been previously shown that reduction of ATP concentration in the recording pipette increases the mean bump amplitude. This is mainly due to inhibition of DAG kinase activity, leading to accumulation of PLC products. This manipulation enhances light excitation of the channels in mutant flies via these products (Hardie et al., 2002). Strikingly, lowering the ATP concentration in the recording pipette caused the reappearance of dark bumps in a dose-dependent manner in the *Gβe1/+* mutant, despite the low Ca^{2+} conditions (Fig. 2E). The reappearance of dark bumps under low Ca^{2+} conditions strongly supports the notion that GDP-GTP exchange at the $\text{G}_q\alpha$ subunit is a Ca^{2+} -independent process and continues under low Ca^{2+} conditions. This result indicates that low Ca^{2+} suppresses dark bump production downstream of $\text{G}_q\alpha$.

Figure 2 clearly shows that dark bump production is Ca^{2+} dependent. However, in light of Figure 2E, it is unclear whether all spontaneous $\text{G}_q\alpha$ activations are manifested in bump production under physiological conditions (i.e., at ~ 1.5 mM external Ca^{2+}). Accordingly, we examined whether the rate of dark bumps is already attenuated under normal Ca^{2+} conditions, despite much higher spontaneous $\text{G}_q\alpha$ activation. To this end, we used dark bump rate following removal of ATP from the recording pipette, as a sensitive bioassay for estimating the actual spontaneous $\text{G}_q\alpha$ activation in WT flies. Strikingly, the observed dark

bump rate increased with time after whole-cell formation, with ATP removed from the recording pipette and reached a bump rate of $\sim 10 \text{ s}^{-1}$ after 40 s (Fig. 4A,C). In contrast, when the recording pipette contained 4 mM ATP, no change in bump rate was observed (Fig. 4B,C). To ensure that the high rate of dark bumps observed in WT flies, under low ATP conditions, was a consequence of spontaneous $\text{G}_q\alpha$ activation, we recorded dark bumps from the *Gβe1/+* mutant fly under reduced ATP conditions as well. Importantly, only scarce dark bumps were observed in the *Gβe1/+* mutant, indicating that in WT flies the high bump rate under reduced ATP conditions is $\text{G}_q\alpha$ dependent (Fig. 4A, bottom, B,C).

To further support the notion that the rate of spontaneous $\text{G}_q\alpha$ activation is higher than the observed dark bump rate under normal Ca^{2+} conditions, we applied an additional strategy. Figure 3 already showed that elevation of cellular $[\text{Ca}^{2+}]$ induced the appearance of dark bumps under low Ca^{2+} conditions. We therefore examined whether increasing cellular $[\text{Ca}^{2+}]$ under 1.5 mM external Ca^{2+} concentration conditions would further increase dark bump rate. Indeed, Figure 4, D and E, shows that bump rate was doubled by increasing cellular $[\text{Ca}^{2+}]$, thus further supporting the above notion. Moreover, in the *Gβe1/+* mutant, increasing cellular $[\text{Ca}^{2+}]$ via the recording pipette did not result in the appearance of dark bumps (Fig. 4D,E). An additional way to temporarily increase cellular $[\text{Ca}^{2+}]$ is obtained by replacing a low Ca^{2+} external solution with a 1.5 mM Ca^{2+} containing solu-

Figure 3. Under low external Ca^{2+} conditions, elevating Ca^{2+} concentration in the patch-pipette solution results in reappearance of dark bumps in a dose-dependent manner. **A**, Whole-cell recordings from WT photoreceptors at total darkness and under low external Ca^{2+} conditions under various concentrations of Ca^{2+} in the recording pipette. **B**, Histogram plotting the mean bump rate of dark bumps at the different conditions. Note that the bump rate increased in a dose-dependent manner ($n = 5$, mean $\pm$ SEM).

tion, which temporarily modifies the equilibrium of the Na^+ - Ca^{2+} exchanger. The external Ca^{2+} manipulations resulted in reverse action of the Na^+ - Ca^{2+} exchanger, transporting Ca^{2+} into the cell, as evidenced by a small outward Na^+ - Ca^{2+} exchange current (Fig. 4F) (Hardie, 1995). This procedure largely increased dark bump rate and strongly suppressed single-photon detection, as evidenced by the virtual inability to discriminate between quantum bumps and dark bumps (Fig. 4F).

Importantly, these observations indicate that although the rate of spontaneous $\text{G}_q\alpha$ activation is at least 10 s^{-1} , this high rate is not manifested in a comparably high rate of dark bump production under physiological conditions. Therefore, we set out to decipher the mechanism underlying suppression of dark bump production under physiological conditions, despite the high rate of spontaneous $\text{G}_q\alpha$ activation.

Low Ca^{2+} suppressed light excitation of the $\text{G}_q\alpha^I$ mutant

The dark bumps are produced by activation of single $\text{G}_q\alpha$ molecules and are characterized by relatively small amplitude and low Ca^{2+} suppression (Fig. 2). The light-induced bumps of the $\text{G}_q\alpha^I$ mutant are known to have small amplitudes and are also produced by activation of single $\text{G}_q\alpha$ molecules (Hardie et al., 2002). We therefore sought to examine whether light induced single $\text{G}_q\alpha$ activation is also suppressed by low Ca^{2+} conditions. To this end we measured the intensity response function (R - $\log I$ curve) of WT and $\text{G}_q\alpha^I$ mutant flies with and without Ca^{2+} in the external solution (Fig. 5A). Knowing that the macroscopic response to light is a summation of quantum bumps (Wong et al., 1980;

Barash and Minke, 1994) implies that if bumps of $\text{G}_q\alpha^I$ mutant flies cannot be produced under low Ca^{2+} conditions, one would expect a highly reduced macroscopic response to light in this mutant under these low Ca^{2+} conditions. Figure 5 shows that this expectation was realized. Although the $\text{G}_q\alpha^I$ mutant shows highly reduced response amplitude at any light intensity because of its highly reduced $\text{G}_q\alpha$ expression level (Fig. 5C; Scott et al., 1995), it still produces a considerable response to intense lights (Fig. 5A,B). Strikingly, at low external $[\text{Ca}^{2+}]_i$, the macroscopic response to light in this mutant was nearly abolished, showing highly reduced amplitude even when the stimulating light intensity was an order of magnitude higher (Fig. 5A, inset). Addition of 1 mM Ca^{2+} into the recording pipette partially rescued the light response under low Ca^{2+} conditions (Fig. 5A,B). In contrast, removing external Ca^{2+} largely enhanced the amplitude of the light-induced current (LIC) of WT flies (Fig. 5B), mainly due to removal of open channel block (Hardie and Mojet, 1995; Parnas et al., 2007). This result demonstrates that light excitation is preserved in WT flies under low Ca^{2+} conditions. Figures 2 and 5 thus indicate that active single $\text{G}_q\alpha$ molecules, when either activated spontaneously in WT flies or by light in the $\text{G}_q\alpha^I$ mutant are virtually eliminated under low Ca^{2+} conditions.

Reduction of PLC expression levels or its catalytic activity results in suppression of light excitation under low Ca^{2+} conditions: a working hypothesis

The selective abolishment of active single $\text{G}_q\alpha$ molecules under low Ca^{2+} conditions can be explained by assuming that a critical level of PLC activity is required to produce a bump. Therefore, the Ca^{2+} dependence of bump production arises from Ca^{2+} regulation of PLC catalytic activity. When PLC reaches a minimal activity level, it triggers synchronous channel activation downstream of PLC leading to bump production (Pumir et al., 2008). According to this hypothesis, sporadic activation of single $\text{G}_q\alpha$ molecules in the dark, followed by activation of a single PLC molecule, would fail to induce dark bump formation under low Ca^{2+} conditions even though dark bumps would be expected to form under normal Ca^{2+} conditions (Fig. 6A). This failure, under low Ca^{2+} conditions, results from reduced PLC catalytic activity when the availability of cytosolic Ca^{2+} is reduced (Toyoshima et al., 1990; Running Deer et al., 1995; Hardie, 2005). On the other hand, photon activation of rhodopsin simultaneously activates several (~ 5) neighboring $\text{G}_q\alpha$ molecules within ~ 100 ms, followed by activation of several neighboring PLC molecules (Fig. 6A; Hardie et al., 2002). This photon activation integrates the relatively low activity level of single PLC molecules under low Ca^{2+} conditions and exceeds the minimal level of integrated PLC activity that is required for synchronous channel activation and resulting in bump production, even under low Ca^{2+} conditions.

To test this notion we examined 3 cases of reduced integrated PLC activity. A relatively low level of integrated cellular PLC activity can arise from several independent causes: (1) reduction in PLC expression levels due to specific mutations (Pearn et al., 1996); (2) low catalytic activity of PLC due to low $[\text{Ca}^{2+}]_i$ (Toyoshima et al., 1990); or (3) low catalytic activity of PLC due to mutation of the enzyme catalytic site (Yoon et al., 2004).

A reduction of PLC expression strongly suppresses light excitation under low Ca^{2+} conditions

We first tested the PLC deficient mutant *norpa*^{P57}, showing normal $\text{G}_q\alpha$ (Fig. 5C) but reduced PLC expression levels (Fig. 6B). The *norpa*^{P57} mutant, carries a single missense mutation (Table

Figure 4. Dark bump activity is dramatically increased when omitting ATP from the recording pipette or by increasing intracellular Ca^{2+} . **A**, Dark bump activity from WT and *Gqα¹* mutant flies using 1.5 mM Ca^{2+} containing extracellular solution and pipette solution with no ATP. Note that in WT flies both the rate and amplitude are increased through time, while in *Gqα¹* mutant flies dark bumps are scarce. **B**, Histogram plotting the mean bump rate with 4 mM ATP and without ATP in the recording pipette of WT and *Gqα¹* mutant flies (the color code applies also to C and D, $n = 4$, mean $\pm$ SEM, $**p < 0.01$; ns, not significant, $p > 0.05$). **C**, Histogram plotting the mean instantaneous dark bump rate with 4 mM ATP and without ATP of 10 s intervals ($n = 4$, mean $\pm$ SEM). Note the increase in the mean instantaneous dark bump rate in WT flies when ATP is omitted from the recording pipette solution while, no change in the instantaneous dark bump rate when 4 mM ATP is added to the recording pipette solution. **D**, Dark bump rate is increased when adding 1 mM Ca^{2+} to the recording pipette solution under 1.5 mM external Ca^{2+} conditions compared with normal pipette solution. **E**, Histogram plotting the mean bump rate with 1 mM and without Ca^{2+} in the recording pipette ($n = 4$, mean $\pm$ SEM, $**p < 0.01$; ns, not significant, $p > 0.05$). **F**, Whole-cell recordings from WT flies under low Ca^{2+} condition and the effect of perfusion with Ca^{2+} containing extracellular solution. Quantum bumps produced by light stimulation of 10 EP/s at both conditions is also demonstrated. Note that no dark activity is seen under low Ca^{2+} conditions while a response to light stimulation is observed. In extracellular solution containing 1.5 mM Ca^{2+} , dark bumps appear at a higher than normal rate and a light response can hardly be detected. Also note that addition of 1.5 mM Ca^{2+} induced a small outward current due to temporary reverse activation of the electrogenic $\text{Na}^+ - \text{Ca}^{2+}$ exchanger, which transports Ca^{2+} into the cell.

3), in the C2 domain of PLC. This mutation largely affects the PLC protein expression level ($\sim 13\%$ of WT, Fig. 6B), but it also may affect PLC catalytic activity (Pearn et al., 1996). In the *norpa^{P57}* mutant at 1.5 mM external Ca^{2+} , dark bump activity was observed with a similar rate and a slight (25%) reduction of mean peak amplitude compared with WT (Fig. 6C). Accordingly, the

appearance of dark bumps in the *norpa^{P57}* mutant implies that the reduced PLC catalytic activity is still high enough to allow bump production by single $\text{G}_q\alpha$ activation at 1.5 mM external $[\text{Ca}^{2+}]$. Moreover, low Ca^{2+} conditions abolished the appearance of dark bumps, while the addition of 1 mM Ca^{2+} into the recording pipette resulted in the reappearance of the dark bumps (Fig. 6C) similar to WT (Figs. 2B, 3). Nevertheless, this mutant shows significant reduction in mean amplitude of light-induced bumps (Hardie et al., 2002). Therefore, we examined the effect of reduced external $[\text{Ca}^{2+}]$ on the $R\text{-log}I$ curve of this mutant relative to WT flies (Fig. 6D,E). In contrast to WT flies (Fig. 5A,B), in the *norpa^{P57}* mutant, removal of external Ca^{2+} from the bathing solution dramatically reduced the response amplitudes at all light intensities and shifted the $R\text{-log}I$ curve to much higher light intensities. On the other hand, addition of 1 mM Ca^{2+} to the recording pipette under low Ca^{2+} conditions rescued the effect of external Ca^{2+} removal in this mutant (Fig. 6D,E), thus showing that Ca^{2+} is a limiting factor for inducing the response to light under these conditions.

Low catalytic activity of PLC due to a mutation in the catalytic site eliminates dark bumps in the presence of Ca^{2+} and strongly suppresses light excitation in the absence of Ca^{2+}

To support the notion that a critical level of PLC catalytic activity is required for production of a dark bump, we used another PLC defective mutant, the *norpa^{H43}*. This mutant features normal $\text{G}_q\alpha$ expression levels (Fig. 5C) and modestly reduced PLC expression levels (Fig. 6B), yet highly reduced PLC catalytic activity compared with WT (Yoon et al., 2004). The *norpa^{H43}* mutant fly, carries two missense mutations (Table 3), which are located at the X-box catalytic domain and close to the C-terminal end of the protein, respectively. In contrast to WT and the *norpa^{P57}* mutant, the *norpa^{H43}* mutant barely exhibited dark bumps either in 1.5 mM Ca^{2+} containing extracellular solution or in the same extracellular solution with 1 mM Ca^{2+} in the recording pipette (Fig. 7A). This result indicates that a critical level of PLC activity is required for dark bump production. Moreover, the

amplitude and rate of the light-induced bumps in *norpa^{H43}* flies were highly reduced compared with WT (Fig. 7B,C). Figure 7C shows an amplitude distribution histogram of dark bumps and quantum bumps of WT vs quantum bumps of the *norpa^{H43}* mutant. The quantum bumps of the *norpa^{H43}* mutant were similar in size to WT dark bumps and their rate was low

Figure 5. The $G_q\alpha^1$ mutant fly shows highly reduced sensitivity to light at low Ca^{2+} compared with 1.5 mM Ca^{2+} conditions. **A**, Intensity–response (R –log I) relationship of WT (triangles) and $G_q\alpha^1$ mutant (circles) flies under low Ca^{2+} , 1.5 mM Ca^{2+} and under low Ca^{2+} conditions with 1 mM Ca^{2+} in the recording pipette solution. Note that the WT (R –log I) relationship was shifted to the left when measured under low Ca^{2+} conditions compared with 1.5 mM Ca^{2+} while, R –log I relationship of the $G_q\alpha^1$ mutant was shifted to the right. Addition of 1 mM Ca^{2+} to the recording pipette solution partially rescued the low sensitivity of the $G_q\alpha^1$ under low Ca^{2+} conditions. Moreover, note that at extremely high intensities of 1.5×10^7 EP/s under low Ca^{2+} condition the response amplitude of $G_q\alpha^1$ photoreceptors is very small. Inset: Intensity–response (log R –log I) relationship ($n = 5$, mean $\pm$ SEM). **B**, A representative light-induced response of WT and $G_q\alpha^1$ mutant to 10^3 and 10^6 EP/s respectively, under 1.5 mM Ca^{2+} , low Ca^{2+} and under low Ca^{2+} conditions with 1 mM Ca^{2+} in the recording pipette solution. **C**, Western blot analysis of heads homogenate of dark raised WT, $G_q\alpha^1$, $G_q\alpha^1/+$ and $norPA$ mutant alleles ($norPA^{P24}$, $norPA^{P57}$, $norPA^{H43}$) using specific *Drosophila* antibodies for $G_q\alpha$ and MOESIN as indicated. Bottom panel shows a histogram plotting the relative density of the corresponding $G_q\alpha$ bands of the different fly strains ($n = 3$, mean $\pm$ SEM).

(7.6 pA, 1.7 bumps/s in the $norPA^{H43}$ mutant, 7.4 pA in WT dark and 22.6 pA, 6.6 bumps/s in WT light). The low rate of bump production in the PLC mutant indicates that there is a low probability that photon activation of several PLC molecules would reach the minimal PLC catalytic activity level required for bump production (see model in Fig. 6A). The similarity in bump amplitude of mutant quantum bumps and WT dark bumps indicates that the integrated PLC activity of several mutant PLCs can reach the catalytic activity level of a single WT PLC molecule. These results strongly support the notion that a critical level of PLC activity is required for any kind of bump production.

These findings were further corroborated by measuring the Ca^{2+} dependence of the macroscopic response to light of the $norPA^{H43}$ mutant. In this mutant, removing external Ca^{2+} from the bathing solution dramatically reduced the amplitude of the LIC at all light intensities and shifted the R –log I curve to much higher light intensities (Fig. 7D). Addition of 1 mM Ca^{2+} into the pipette under low external Ca^{2+} conditions, rescued the LIC, thus indicating that Ca^{2+} is a limiting factor for maintaining excitation in this mutant (Fig. 7E). Hence, Figure 7, D and E, shows that further reduction of the already low mutant PLC catalytic activity via Ca^{2+} reduction totally abolished light excitation. This result is also consistent with the requirement for a minimal level of PLC catalytic activity to produce a quantum bump.

Dark bump suppression by replacing Ca^{2+} with Mg^{2+} , Sr^{2+} or Ba^{2+}

The regulation of PLC catalytic activity by Ca^{2+} has been thoroughly investigated. These studies show that the positive charge of Ca^{2+} is used to counterbalance local negative charges formed in the active site during the course of the catalytic reaction. Accordingly, Ca^{2+} performs electrostatic stabilization of both the substrate and the transition state, thus providing a twofold contribution to lower the activation energy of the enzyme reaction (Essen et al., 1997). However, only a few studies have addressed the cation selectivity of PLC catalytic activity. Nevertheless, these studies have indicated that replacing Ca^{2+} with other group IIA divalent cations (e.g., Mg^{2+} , Sr^{2+} and Ba^{2+}) reduces the catalytic activity of the enzyme (Schwartz et al., 1987; Yotsushima et al., 1993).

Several stages of bump generation require Ca^{2+} . Therefore, it is unclear whether low Ca^{2+} conditions affect bump production at the PLC level by reducing its catalytic activity. To isolate a Ca^{2+} specific stage, it might be useful to examine the effects of other divalent cations on dark bump production. We therefore examined the effect of Ca^{2+} substitution with Mg^{2+} , Sr^{2+} or Ba^{2+} on dark bump rate. Strikingly, none of these ion substitutions induced dark bump activity (Fig. 8A, B), consistent with previously described reduced PLC catalytic activity in the presence of these cations. Therefore, Figures 7 and 8 together indicate that Ca^{2+}

Figure 6. The *norpA*^{P57} hypomorph show highly reduced light sensitivity under low Ca²⁺ conditions compared with 1.5 mM Ca²⁺. **A**, A model of the initial stages of phototransduction underlying dark bump production (top) and quantum bump production (bottom). **B**, Western blot analysis of heads homogenate of dark raised WT, *norpA* mutant alleles (*norpA*^{P24}, *norpA*^{P57}, *norpA*^{H43}) and *Gαq*¹ mutant using specific *Drosophila* antibodies for NORPA and MOESIN as indicated. Bottom panel shows a histogram plotting the relative density of the corresponding NORPA bands of the different fly strains (*n* = 3, mean ± SEM). **C**, Dark bumps are observed in *norpA*^{P57} at total darkness under 1.5 mM Ca²⁺ conditions (top trace). Low Ca²⁺ condition abolished the dark activity (middle trace). Addition of 1 mM Ca²⁺ to the recording pipette solution resulted in the reappearance of dark bumps under low Ca²⁺ conditions (bottom trace) (*n* = 4). **D**, Intensity–response (*R*–log*I*) relationship of *norpA*^{P57} mutant flies under low Ca²⁺, 1.5 mM Ca²⁺ and under low Ca²⁺ conditions with 1 mM Ca²⁺ in the recording pipette solution. Note that under low Ca²⁺ conditions drastically reduced the light response, compared with 1.5 mM Ca²⁺ while addition of 1 mM Ca²⁺ to the recording pipette solution rescued the low sensitivity under low Ca²⁺ conditions. Moreover, note that at extremely high intensities of 1.5 × 10⁷ EP/s under low Ca²⁺ conditions the response amplitude of *norpA*^{P57} photoreceptors are very small. Inset: Intensity–response (log*R*–log*I*) relationship (*n* = 5, mean ± SEM). **E**, A representative light-induced responses of *norpA*^{P57} mutant to 10⁴ EP/s under 1.5 mM Ca²⁺, low Ca²⁺ and under low Ca²⁺ conditions with 1 mM Ca²⁺ in the recording pipette solution.

Table 3. Details of the various alleles used in this study

Allele	Description	Total protein level (% WT)	Specific PLC activity (% WT)	Reference
<i>w¹¹¹⁸</i> (WT)	White eyed			
<i>Gαq¹</i> (<i>Gαq</i> ¹)	Splice acceptor site leading to in-frame deletion of residues 154–156	~2% (<i>Gαq</i> , Fig. 5C)	—	(Scott et al., 1995)
<i>Gαq¹/SM6</i> (heterozygote, <i>Gαq</i> ¹ /+)		~60% (<i>Gαq</i> , Fig. 5C)	—	
<i>Gβe¹/TM6B</i> (heterozygote, <i>Gβe</i> ¹ /+)	Cys293Tyr	~50% (<i>Gβ</i>)	—	(Dolph et al., 1994; Elia et al., 2005)
<i>norpA^{H43};bw</i> ;st abbreviation: <i>norpA^{H43}</i>	Ser347Asn and Thr1007Ser	~79% (PLC, Fig. 6B)	~8%	(Yoon et al., 2004)
<i>norpA^{P57};bw</i> ;st abbreviation: <i>norpA^{P57}</i>	Gly768Asp	~13% (PLC, Fig. 6B)	~27%	(Pearn et al., 1996; Yoon et al., 2004)

facilitates dark bump production by increasing the catalytic activity of PLC.

The Ca²⁺ dependence of quantum bump shape is determined downstream of PLC

The Ca²⁺ dependence of PLC activity, in general, and of fly eye PLC (NORPA) in particular has been reported (Toyoshima et al., 1990; Running Deer et al., 1995; Essen et al., 1997; Hardie, 2005). In *Drosophila*, both *in vivo* and *in vitro* measurements revealed Ca²⁺ dependence of PLC activity. This activity shows a bell-shaped dependence of PIP₂ hydrolysis on [Ca²⁺], with maximal basal activity in the range of 10^{−7}–10^{−5} M [Ca²⁺] (Toyoshima et al., 1990; Running Deer et al., 1995; Hardie, 2005).

Previous studies have shown a strong Ca²⁺-dependent facilitation of bump kinetics (Henderson et al., 2000), which may arise from either a facilitation of PLC activity or from a Ca²⁺-dependent increase of channel sensitivity to a PLC product (Hardie and Postma, 2008). The study of the *norpA*^{H43} mutant revealed an important phenomenon: Although the PLC catalytic activity in this mutant is very low, the waveform and time to peak of the quantum bumps were virtually unaffected by the mutation (Fig. 9*A,B*). This result clearly demonstrates that the effect of low Ca²⁺ on response kinetics is PLC independent and operates downstream of PLC activity. This conclusion was further supported by experiments in which Ca²⁺ was replaced by Sr²⁺ and Ba²⁺ (Fig. 9*C,D*). Although dark bump production was suppressed under these conditions, quantum bump kinetics were

Figure 7. The *norpA^{H43}* mutant with low PLC catalytic activity shows no dark bumps, reduced quantum bump rate and amplitude, and a highly reduced sensitivity to light under low Ca^{2+} conditions. **A**, Dark bumps are not observed in *norpA^{H43}* at total darkness under 1.5 mM Ca^{2+} conditions (top trace) and under 1.5 mM Ca^{2+} conditions with the addition of 1 mM Ca^{2+} to the patch-pipette solution (bottom trace) ($n = 4$). **B**, Light-induced bumps in *norpA^{H43}* mutant flies in response to light intensity of 10 EP/s (top trace) and in WT flies to light intensities of 10 EP/s (middle trace) and 1 EP/s (bottom trace) ($n = 4$). Note that the light-induced bumps of the mutant are small, while their kinetics are as fast as those of WT bumps. **C**, Histogram plotting distribution of bump amplitude of WT dark bumps, WT quantum bumps and *norpA^{H43}* quantum bumps ($n = 117$). Inset, Gaussian fits to the amplitude distribution histograms (mean peak amplitude; WT dark = 7.4 pA, WT light = 22.6 pA and *norpA^{H43}* light = 7.6 pA). **D**, Intensity–response (R – $\log I$) curve of *norpA^{H43}* mutant flies under low Ca^{2+} , 1.5 mM Ca^{2+} and under low Ca^{2+} conditions with 1 mM Ca^{2+} in the recording pipette solution. Note that even at extremely high intensities of 1.5×10^7 EP/s under low Ca^{2+} conditions the response amplitude of *norpA^{H43}* photoreceptors was very small. Inset, Intensity–response ($\log R$ – $\log I$) relationship ($n = 5$, mean $\pm$ SEM). **E**, A representative light-induced response to 10^6 EP/s in *norpA^{H43}* mutant under low Ca^{2+} , 1.5 mM Ca^{2+} , and under low Ca^{2+} conditions with 1 mM Ca^{2+} in the recording pipette solution.

only slightly slowed down. The fact that Sr^{2+} or Ba^{2+} substitute Ca^{2+} in accelerating bump kinetics, but do not substitute Ca^{2+} in rescuing dark bump generation is important because it strongly suggests that a second site of Ca^{2+} action exists. In contrast to Sr^{2+} and Ba^{2+} , substituting Ca^{2+} with Mg^{2+} drastically slowed down the kinetics of the quantum bumps and macroscopic response (Figs. 8C, 9C). This result suggests that unlike Sr^{2+} and Ba^{2+} , Mg^{2+} cannot substitute Ca^{2+} in accelerating bump kinetics. Altogether, these observations strongly suggest that Ca^{2+} operates in at least two stages of bump production: (1) as a unique cofactor in PLC catalytic activity that cannot be replaced by other divalent cations; (2) as a factor required for the fast kinetics of synchronous channel activation that operates downstream of PLC and can be substituted by Sr^{2+} and Ba^{2+} but not by Mg^{2+} .

Discussion

Drosophila flies are mainly active during medium intensities of ambient lights at dusk and dawn (Rieger et al., 2007). At these light intensities, arriving photons are absorbed by different microvilli of a single photoreceptor cell. Each of these $\sim 30,000$ microvilli functions as an independent unit, producing a single quantum bump, while the cell membrane integrates all bumps

(Hardie and Raghu, 2001). A high rate of dark bumps, results in their temporal summation, which may reach quantum bump amplitude and hamper single-photon detection (Figs. 2B, arrows, 4F). A similar example of spontaneous G-protein activation comes from a study in yeasts, showing receptor independent spontaneous G-protein activation when a GAP was genetically eliminated. This leads to spontaneous activation of the G-protein-mediated mating pathway at levels normally seen upon receptor activation (Siekhaus and Drubin, 2003). Accordingly, suppression of the G-protein-dependent spontaneous activation of mating in yeasts occurs by accelerating the GTPase reaction. The two studies show that the high fidelity of G-protein-mediated signaling systems relies on suppression of background noise induced by receptor independent activity of the G-proteins.

Suppression of dark noise by a PLC-mediated mechanism

In this work we show that in *Drosophila* under physiological conditions the mechanism underlying false signaling suppression is partially concealed but can be readily exposed by mutations or nonphysiological conditions. Using the single-photon responses and dark bumps of *Drosophila* mutant flies facilitated the discovery of the mechanism underlying suppression of dark noise at the single molecule level *in vivo*. The mechanism underlying dark

Figure 8. Group IIA divalent cations Mg^{2+} , Sr^{2+} , and Ba^{2+} do not induce dark bumps. **A**, Whole-cell recordings from WT photoreceptors at total darkness at the various conditions as indicated. Note that only Ca^{2+} induced dark bump activity. **B**, Histogram plotting the mean bump rate of dark bumps at the different conditions ($n = 4$, mean $\pm$ SEM). **C**, Light response to 1EP/s intensity. Note that in all cases bumps can be induced by the light stimuli. **D**, Histogram plotting the mean bump rate of light-induced bumps at the different conditions ($n = 4$, mean $\pm$ SEM).

bump suppression and reliable quantum bump production relies on the requirement for a crucial level of PLC activity that is necessary for bump production. Accordingly, activation of a single PLC molecule has a low probability of producing a bump, as manifested by the low rate of dark bumps even when a high rate of spontaneous $\text{G}_q\alpha$ activation exists. On the other hand, photon-induced synchronized activation of several neighboring $\text{G}_q\alpha$ molecules, which activate several PLC molecules, has a high probability of producing a bump. This mechanism leads to at least 85% suppression of dark bump activity under physiological conditions.

The crucial finding, which supports the notion that a certain minimal level of integrated PLC catalytic activity is required for bump production came from the study of the *norPA*^{H43}. This mutant has nearly normal PLC expression levels but ~10-fold reduction in enzymatic catalytic activity relative to WT. In this mutant there is no production of dark bumps and both the rate and amplitude of the quantum bumps are highly reduced. Interestingly, the amplitude distribution of the mutant quantum bumps was similar to that of WT dark bumps (Fig. 7C). These observations show that the level of PLC catalytic activity is the critical parameter for triggering bump production because the only difference between the mutant and WT flies is the mutant's reduced catalytic activity. Accordingly, the activated single mutant PLC molecule with low catalytic activity is unable to reach the required minimal level of enzymatic activity that is required for bump production, resulting in total suppression of dark bumps. Importantly, several light activated mutant PLC molecules, each with low catalytic activity as in-

dividual unit yet synchronously activated as a group, can reach the minimal level required for bump production albeit with low probability. Therefore, a reduction in both the rate and amplitude of the quantum bumps is observed under such circumstances.

An additional way to reduce PLC catalytic activity can be obtained by reducing cellular $[\text{Ca}^{2+}]$, which eliminates dark bump production (Fig. 2A). In WT flies, under low Ca^{2+} conditions, light excitation is still maintained and quantum bumps are preserved because the integrated PLC catalytic activity is sufficient to reach the critical level for bump production. However, in the *G_q\alpha*¹ and *norPA* mutants, light excitation is lost at low $[\text{Ca}^{2+}]$ because the integrated PLC catalytic activity is further reduced by the low $[\text{Ca}^{2+}]$. This low PLC catalytic activity does not exceed the required level for bump productions.

An alternative strategy to reduce dark noise and maintain signal reliability can be obtained by large amplification of the signal. In vision, this strategy has a major drawback because the system's operating range is reduced by the large amplification. The relatively small gain (~5-fold) between photopigment activation and the G-protein stage, which is maintained throughout the fly phototransduction cascade up to channel openings (*Drosophila* and *Musca*; Minke

and Stephenson, 1985; Hardie et al., 2002), maintains a balance between a wide operating range required for vision and noise suppression. Hence, the existence of a nonlinearity between PLC activity and bump production enables dark noise suppression in a low gain sensory system.

Suppression of dark noise by compartmentalization

An additional mechanism that suppresses the dark noise is the specialized structure of the signaling compartment. The signaling compartment, the rhabdomere, is composed of ~30,000 tightly packed microvilli constituting an integral part of the plasma membrane, where the proteins of the phototransduction machinery reside. The confinement of the phototransduction machinery into identical and separated topological segments, divides the extremely high protein expression level into diminutive amounts of functional groups. This division reduces the possibility of integration and suppresses dark noise. By adding a "gate keeper", in the form of nonlinearity between PLC activity and bump production, spontaneous activity can be further reduced while concomitantly increasing the signal-to-noise ratio. The functional segregation of individual microvilli was revealed in the *G_q\alpha*¹, *norPA*^{P57} and *norPA*^{H43} mutants under low Ca^{2+} conditions, when responses to light were virtually eliminated. This response suppression was apparent even at extremely high light intensities of 1.5×10^7 effective photons s^{-1} , where ~50 rhodopsin molecules are activated within each microvillus. One could expect that a summation of PLC activities among different microvilli may lead to a bump-like response, even if the activity within a single microvillus is below the critical PLC activity level

Figure 9. Bump kinetics is independent of PLC catalytic activity. **A**, Whole-cell recordings from WT photoreceptors in total darkness (top trace), light-induced bumps in WT in response to light intensity of 1 EP/s (middle trace) and light-induced bumps in *norpA^{H43}* mutant flies in response to light intensity of 10 EP/s (bottom trace). **B**, Histogram plotting the time to peak (t_p) distribution of WT dark bumps, WT quantum bumps, and *norpA^{H43}* quantum bumps ($n = 118$). Inset left, Gaussian fits to the t_p distribution histograms (mean t_p : WT dark = 14.6 ms, WT light = 16.4 ms and *norpA^{H43}* light = 16.8 ms). Inset right, Normalized average of 118 bumps from traces in A (t_p : WT dark = 14.7 ms, WT light = 16.4 ms and *norpA^{H43}* light = 16.1 ms). **C**, Light response to a brief 50 ms flash at the various group IIA divalent cations solutions as indicated (arrows indicate the t_p of the light response, t_p : Low Ca^{2+} = 189 ms, 1.5 mM Ca^{2+} = 118 ms, 1.5 mM Mg^{2+} = 335 ms, 1.5 mM Sr^{2+} = 141 ms, and 1.5 mM Ba^{2+} = 128 ms). **D**, Normalized average of 100 bumps at the presence of various group IIA divalent cations solutions as indicated (arrows indicate the t_p of the averaged quantum bump, t_p : Low Ca^{2+} = 71.5 ms, 1.5 mM Ca^{2+} = 16 ms, 1.5 mM Sr^{2+} = 25.7 ms, 1.5 mM Ba^{2+} = 30.6 ms and 1.5 mM Ca^{2+} + 4 mM Mg^{2+} = 17.2 ms).

necessary for bump generation. However, contrary to this expected summation, the observed response suppression in the mutants indicates a lack of summation of PLC activity among neighboring microvilli. These findings are consistent with previous suggestion that a microvillus is the minimal functional unit for producing a bump (Hamdorf and Kirschfeld, 1980; Hardie et al., 2002; Yau and Hardie, 2009).

Ca^{2+} dependence of bump kinetics operates downstream of PLC activity

The available data clearly indicate that the discrete nature of the bump does not arise from properties of light activated channels, because direct activation of the channels under physiological conditions by polyunsaturated fatty acid (Chyb et al., 1999) induce channel noise but not bump noise (Hardie and Minke, 1994). A question thus arises as to the mechanism underlying synchronized channel activity that produces the bump. Several observations in the current study give clues, which help identify the phototransduction stages in which synchronized channel activity is produced. Since Ca^{2+} is a major factor in regulation of phototransduction, a study of the Ca^{2+} dependence of bump kinetics, which is determined by the degree of synchronization in channel activation, may shed light on this issue.

The Ca^{2+} dependence of quantum bump amplitude and kinetics have several properties, including two phase initiation and very slow kinetics under low Ca^{2+} conditions (Henderson et al., 2000; Figs. 2, 9). Moreover, the kinetics and amplitude of the averaged bump are largely constant in a large range of external $[\text{Ca}^{2+}]$ between 100 μM and 1.5 mM (Henderson et al., 2000). A major question arises as to how and at what stage of phototransduction does Ca^{2+} regulate bump kinetics? Important clues have emerged from comparison of quantum bump kinetics between the *norpA^{H43}* mutant and WT flies, and from assays on the effects of group IIA divalent cations (Figs. 7–9). Strikingly, the kinetics of the quantum bumps recorded from the *norpA^{H43}* mutant was virtually normal (Fig. 9). If Ca^{2+} affects the bump kinetics at the stage of PLC activity, one would expect significantly slower bump kinetics in the mutant relative to WT when PLC catalytic activity is reduced by the *norpA^{H43}* mutation. However, our observations were to the contrary (Figs. 7, 9). This result indicates that Ca^{2+} affect bump kinetics downstream of PLC. The observation that quantum bump kinetics is virtually normal in the presence of Sr^{2+} and Ba^{2+} , while dark bumps are eliminated (Figs. 8, 9) further support the conclusion that Ca^{2+} affects bump kinetics downstream of PLC. This is because the elimination of dark bumps in the presence of Sr^{2+} and Ba^{2+} is explained by reduced PLC catalytic activity, below the crucial level required for bump

production. The accelerated kinetics of these quantum bumps relative to low Ca^{2+} conditions despite reduced PLC catalytic activity indicates that Sr^{2+} and Ba^{2+} can substitute for Ca^{2+} downstream of PLC. Accordingly, this study shed light on two different sites of Ca^{2+} action in the bump generating mechanism: (1) a site in which Ca^{2+} regulated PLC catalytic activity, (2) a site operating downstream of PLC, most likely at the channel stage. This mechanism determines the degree of synchronized channel activity in a Ca^{2+} -dependent manner.

References

- Barash S, Minke B (1994) Is the receptor potential of fly photoreceptors a summation of single-photon responses? *Comments Theoretical Biology* 3:229–263.
- Bloomquist BT, Shortridge RD, Schneuwly S, Perdew M, Montell C, Steller H, Rubin G, Pak WL (1988) Isolation of a putative phospholipase C gene of *Drosophila*, *norpA*, and its role in phototransduction. *Cell* 54:723–733.
- Chyb S, Raghu P, Hardie RC (1999) Polyunsaturated fatty acids activate the *Drosophila* light-sensitive channels TRP and TRPL. *Nature* 397:255–259.
- Cook B, Bar-Yaacov M, Cohen Ben-Ami H, Goldstein RE, Paroush Z, Selinger Z, Minke B (2000) Phospholipase C and termination of G-protein-mediated signalling *in vivo*. *Nat Cell Biol* 2:296–301.
- Delgado R, Bacigalupo J (2009) Unitary recordings of TRP and TRPL channels from isolated *Drosophila* retinal photoreceptor rhabdomeres: activation by light and lipids. *J Neurophysiol* 101:2372–2379.
- Devary O, Heichal O, Blumenfeld A, Cassel D, Suss E, Barash S, Rubinstein CT, Minke B, Selinger Z (1987) Coupling of photoexcited rhodopsin to inositol phospholipid hydrolysis in fly photoreceptors. *Proc Natl Acad Sci U S A* 84:6939–6943.
- Dolph PJ, Man-Son-Hing H, Yarfitz S, Colley NJ, Deer JR, Spencer M, Hurley JB, Zuker CS (1994) An eye-specific Gb subunit essential for termination of the phototransduction cascade. *Nature* 370:59–61.
- Elia N, Frechter S, Gedi Y, Minke B, Selinger Z (2005) Excess of Gb over G_{α} *in vivo* prevents dark, spontaneous activity of *Drosophila* photoreceptors. *J Cell Biol* 171:517–526.
- Essen LO, Perisic O, Katan M, Wu Y, Roberts MF, Williams RL (1997) Structural mapping of the catalytic mechanism for a mammalian phosphoinositide-specific phospholipase C. *Biochemistry* 36:1704–1718.
- Frechter S, Elia N, Tzarfaty V, Selinger Z, Minke B (2007) Translocation of Gq mediates long-term adaptation in *Drosophila* photoreceptors. *J Neurosci* 27:5571–5583.
- Gu Y, Oberwinkler J, Postma M, Hardie RC (2005) Mechanisms of light adaptation in *Drosophila* photoreceptors. *Curr Biol* 15:1228–1234.
- Hamdorf K, Kirschfeld K (1980) Reversible events in the transduction process of photoreceptors. *Nature* 283:859–860.
- Hardie RC (1991) Whole-cell recordings of the light-induced current in dissociated *Drosophila* photoreceptors: evidence for feedback by calcium permeating the light-sensitive channels. *Proc R Soc Lond B* 245:203–210.
- Hardie RC (1995) Photolysis of caged Ca^{2+} facilitates and inactivates but does not directly excite light-sensitive channels in *Drosophila* photoreceptors. *J Neurosci* 15:889–902.
- Hardie RC (2005) Inhibition of phospholipase C activity in *Drosophila* photoreceptors by 1,2-bis(2-aminophenoxy)ethane N,N,N',N' -tetraacetic acid (BAPTA) and di-bromo BAPTA. *Cell Calcium* 38:547–556.
- Hardie RC, Minke B (1994) Spontaneous activation of light-sensitive channels in *Drosophila* photoreceptors. *J Gen Physiol* 103:389–407.
- Hardie RC, Mojet MH (1995) Magnesium-dependent block of the light-activated and *trp*-dependent conductance in *Drosophila* photoreceptors. *J Neurosci* 15:2590–2599.
- Hardie RC, Postma M (2008) Phototransduction in microvillar photoreceptors of *Drosophila* and other invertebrates. In: *The senses: a comprehensive reference* (Allan IB, Akimichi K, Gordon MS, Gerald W, Thomas DA, Richard HM, Peter D, Donata O, Stuart F, Gary KB, Bushnell MC, Jon HK, Esther G, eds), pp 77–130. New York: Academic.
- Hardie RC, Raghu P (2001) Visual transduction in *Drosophila*. *Nature* 413:186–193.
- Hardie RC, Martin F, Cochrane GW, Juusola M, Georgiev P, Raghu P (2002) Molecular basis of amplification in *Drosophila* phototransduction. Roles for G protein, phospholipase C, and diacylglycerol kinase. *Neuron* 36:689–701.
- Henderson SR, Reuss H, Hardie RC (2000) Single photon responses in *Drosophila* photoreceptors and their regulation by Ca^{2+} . *J Physiol Lond* 524:179–194.
- Huang J, Liu CH, Hughes SA, Postma M, Schwenning CJ, Hardie RC (2010) Activation of TRP channels by protons and phosphoinositide depletion in *Drosophila* photoreceptors. *Curr Biol* 20:189–197.
- Katz B, Minke B (2009) *Drosophila* photoreceptors and signaling mechanisms. *Front Cell Neurosci* 3:2.
- Kosloff M, Elia N, Joel-Almagor T, Timberg R, Zars TD, Hyde DR, Minke B, Selinger Z (2003) Regulation of light-dependent G_{α_q} translocation and morphological changes in fly photoreceptors. *EMBO J* 22:459–468.
- Leung HT, Tseng-Crank J, Kim E, Mahapatra C, Shino S, Zhou Y, An L, Doerge RW, Pak WL (2008) DAG lipase activity is necessary for TRP channel regulation in *Drosophila* photoreceptors. *Neuron* 58:884–896.
- Minke B, Stephenson RS (1985) The characteristics of chemically induced noise in *Musca* photoreceptors. *J Comp Physiol* 156:339–356.
- Nikolic K, Loizu J, Degenaar P, Toumazou C (2010) A stochastic model of the single photon response in *Drosophila* photoreceptors. *Integr Biol (Camb)* 2:354–370.
- Oberwinkler J, Stavenga DG (2000) Calcium imaging demonstrates colocalization of calcium influx and extrusion in fly photoreceptors. *Proc Natl Acad Sci U S A* 97:8578–8583.
- Parnas M, Katz B, Minke B (2007) Open channel block by Ca^{2+} underlies the voltage dependence of *Drosophila* TRPL channel. *J Gen Physiol* 129:17–28.
- Parnas M, Katz B, Lev S, Tzarfaty V, Dadon D, Gordon-Shaag A, Metzner H, Yaka R, Minke B (2009) Membrane lipid modulations remove divalent open channel block from TRP-like and NMDA channels. *J Neurosci* 29:2371–2383.
- Pearn MT, Randall LL, Shortridge RD, Burg MG, Pak WL (1996) Molecular, biochemical, and electrophysiological characterization of *Drosophila* *norpA* mutants. *J Biol Chem* 271:4937–4945.
- Peretz A, Suss-Toby E, Rom-Glas A, Arnon A, Payne R, Minke B (1994) The light response of *Drosophila* photoreceptors is accompanied by an increase in cellular calcium: effects of specific mutations. *Neuron* 12:1257–1267.
- Pumir A, Graves J, Ranganathan R, Shraiman BI (2008) Systems analysis of the single photon response in invertebrate photoreceptors. *Proc Natl Acad Sci U S A* 105:10354–10359.
- Rieger D, Fraunholz C, Popp J, Bichler D, Dittmann R, Helfrich-Förster C (2007) The fruit fly *Drosophila melanogaster* favors dim light and times its activity peaks to early dawn and late dusk. *J Biol Rhythms* 22:387–399.
- Running Deer JL, Hurley JB, Yarfitz SL (1995) G protein control of *Drosophila* photoreceptor phospholipase C. *J Biol Chem* 270:12623–12628.
- Schwartz DW, Halverson JB, Palmer JW, Feinberg H (1987) Characterization of phospholipase C-mediated phosphatidylinositol degradation in rat heart ventricle. *Arch Biochem Biophys* 253:388–398.
- Scott K, Becker A, Sun Y, Hardy R, Zuker C (1995) Gq protein function *in vivo*: genetic dissection of its role in photoreceptor cell physiology. *Neuron* 15:919–927.
- Selinger Z, Minke B (1988) Inositol lipid cascade of vision studied in mutant flies. *Cold Spring Harb Symp Quant Biol* 53:333–341.
- Sieghaus DE, Drubin DG (2003) Spontaneous receptor-independent heterotrimeric G-protein signalling in an RGS mutant. *Nat Cell Biol* 5:231–235.
- Toyoshima S, Matsumoto N, Wang P, Inoue H, Yoshioka T, Hotta Y, Osawa T (1990) Purification and partial amino acid sequences of phosphoinositide-specific phospholipase C of *Drosophila* eye. *J Biol Chem* 265:14842–14848.
- Wong F, Knight BW, Dodge FA (1980) Dispersion of latencies in photoreceptors of *Limulus* and the adapting-bump model. *J Gen Physiol* 76:517–537.
- Wu CF, Pak WL (1975) Quantal basis of photoreceptor spectral sensitivity of *Drosophila melanogaster*. *J Gen Physiol* 66:149–168.
- Yau KW, Hardie RC (2009) Phototransduction motifs and variations. *Cell* 139:246–264.
- Yeandle S, Spiegler JB (1973) Light-evoked and spontaneous discrete waves in the ventral nerve photoreceptor of *Limulus*. *J Gen Physiol* 61:552–571.
- Yoon J, Leung HT, Lee S, Geng C, Kim Y, Baek K, Pak WL (2004) Specific molecular alterations in the *norpA*-encoded phospholipase C of *Drosophila* and their effects on electrophysiological responses *in vivo*. *J Neurochem* 89:998–1008.
- Yotsushima K, Mitsui T, Takaoka T, Hayakawa T, Igaue I (1993) Purification and characterization of membrane-bound inositol phospholipid-specific phospholipase C from suspension-cultured rice (*Oryza sativa* L.) cells (identification of a regulatory factor). *Plant Physiol* 102:165–172.