

Excess of $G\beta_e$ over $Gq\alpha_e$ in vivo prevents dark, spontaneous activity of *Drosophila* photoreceptors

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D*rosophila melanogaster* photoreceptor cells are capable of detecting single photons. This utmost sensitivity is critically dependent on the maintenance of an exceedingly low, dark, spontaneous activity of photoreceptor cells. However, the underlying mechanisms of this hallmark of phototransduction are not fully understood. An analysis of the *Drosophila* visual heterotrimeric ($\alpha\beta\gamma$) Gq protein revealed that wild-type *Drosophila* flies have about a twofold excess of $G\beta$ over $G\alpha$ subunits of the visual Gq protein. Studies of $G\beta_e$ mutants

in which the excess of $G\beta$ was genetically eliminated showed dramatic dark, spontaneous activity of the photoreceptor cells, whereas concurrent genetic reduction of the $G\alpha$ subunit, which restored the excess of $G\beta$, abolished this effect. These results indicate that an excess of $G\beta$ over $G\alpha$ is a strategy used in vivo for the suppression of spontaneous activity, thereby yielding a high signal to noise ratio, which is characteristic of the photoreceptor light response. This mechanism could be relevant to the regulation of G protein signaling in general.

Introduction

Many signaling systems use heterotrimeric ($\alpha\beta\gamma$) G proteins to relay signals from heptahelical receptors to downstream effectors. To accomplish signal transduction, G proteins act as conformational sensors of a guanine nucleotide, which is bound to the α subunit. G proteins that are charged with guanosine diphosphate (GDP) are in the inactive state, where the α and the $\beta\gamma$ subunits are associated with each other. Receptor activation accelerates the exchange of bound GDP for free GTP (Cassel and Selinger, 1978) followed by the dissociation of active $G\alpha$ -GTP from $\beta\gamma$ subunits. Hydrolysis of the bound GTP by a GTPase reaction brings the $G\alpha$ subunit back to the inactive state (Cassel et al., 1977), which is characterized by tightly bound GDP and a reassociation with the $\beta\gamma$ complex. To ensure specificity, high effective concentrations, and speed of interaction, the G protein signaling components are usually attached to the membrane domain as peripheral membrane proteins.

Membrane attachment of heterotrimeric G proteins has been extensively investigated, and the effect of lipid modification on membrane localization has been addressed by several studies (Wedegaertner, 1998; Resh, 1999; Chen and Manning, 2001; Kosloff et al., 2002, 2003; Smotryś and Linder, 2004). All G protein α subunits (with the exception of transducin) are palmitoylated, and some are additionally modified by myristoylation.

The α subunits of Gq/G₁₁, including the *Drosophila melanogaster* eye-specific Gq α , as well as Gs α , G₁₂ α , and G₁₃ α are modified only by palmitoylation. The corresponding $\beta\gamma$ subunits undergo isoprenylation of a cysteine residue at the so-called CAAX box of the γ subunit (for review see Wedegaertner, 1998; Resh, 1999; Chen and Manning, 2001). Plasma membrane attachment of the α subunits Gs α and Gq α is dependent on coexpression with the $\beta\gamma$ subunits (Evanko et al., 2000, 2001). Furthermore, the $\beta\gamma$ subunits, having only one membrane attachment signal on the γ subunit, are poorly targeted to the plasma membrane and require coexpression of the α subunit for efficient plasma membrane attachment (Evanko et al., 2001; Michaelson et al., 2002; Takida and Wedegaertner, 2003). Altogether, these studies led to a model of two membrane attachment signals that are needed for plasma membrane attachments and localization of heterotrimeric G protein subunits (Wedegaertner, 1998; Resh, 1999). It should be noted, however, that most of these studies have been performed by using various culture cells that were transfected with vectors yielding overexpressed proteins (usually the α and $\beta\gamma$ subunits of the heterotrimeric G protein). This procedure is bound to cause distortion of the original stoichiometry of α and $\beta\gamma$ subunits, which is difficult to control under these conditions. The extensively studied *Drosophila* visual system combined with the large repertoire of *Drosophila* visual mutants offer a unique opportunity to study in vivo the various roles of the $\beta\gamma$ dimer, its cellular localization, and the functional consequences of altering $\alpha/\beta\gamma$ stoichiometry.

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Abbreviations used in this paper: ERG, electroretinogram; GDP, guanosine diphosphate.

The *Drosophila* visual system is a specialized system that is composed of highly polarized and compartmentalized cells that sequester the phototransduction machinery in a specific signaling compartment called the rhabdomere (Minke and Hardie, 2000; Hardie and Raghu, 2001). This signaling compartment is functionally equivalent to the vertebrate rod photoreceptor outer segment, which also sequesters the phototransduction machinery in a specific cell compartment. Phototransduction in *Drosophila* is initiated upon the activation of rhodopsin by light and proceeds through a photoreceptor-specific Gq protein (Gq_e; Scott et al., 1995), which, in turn, activates the phospholipase C enzyme effector (Devary et al., 1987). Upon activation, the eye-specific Gqα subunit (Gqα_e) dissociates from the eye-specific βγ dimer (Gβγ_e) and translocates, at least in part, from the membrane to the cytosol (Kosloff et al., 2003; Cronin et al., 2004).

In this study, we show (by using a series of eye-specific Gβ_e hypomorph mutants) that the βγ dimer has a crucial role in both membrane attachment and rhabdomeral targeting of the α subunit that can account for the decreased light sensitivity previously observed in these mutants (Dolph et al., 1994). On the other hand, by using the almost null mutant for the eye-specific Gqα subunit *Gaq¹*, we found that the βγ dimer is dependent on the α subunit for membrane attachment but not for targeting to the rhabdomere, suggesting a role for the βγ dimer in targeting the heterotrimer to the photoreceptor signaling compartment (the rhabdomere). An analysis of the protein levels of Gqα_e and Gβ_e subunits revealed a surprising twofold excess of the Gβ_e subunit over the Gqα_e subunit. Mutants that eliminated this excess showed a dramatic increase in spontaneous activity of the

phototransduction cascade. Conversely, double mutations that also reduced the level of Gqα_e and, thereby, restored the excess of Gβ_e over Gqα_e completely reversed this phenotype. Together, these results provide a significant insight into the strategy used by the photoreceptor cell in vivo to avoid spontaneous activity at the G protein level.

Results

The levels of Gqα_e and Gβ_e subunits in *Drosophila* photoreceptors are maintained independently of one another

The α subunit of the heterotrimeric G protein and the tightly associated complex of βγ subunits undergo dissociation and reassociation during activation of the phototransduction cascade. Therefore, it is expected that these subunits would influence one another's level, localization, and function. Previous studies that addressed these questions used tagged subunits

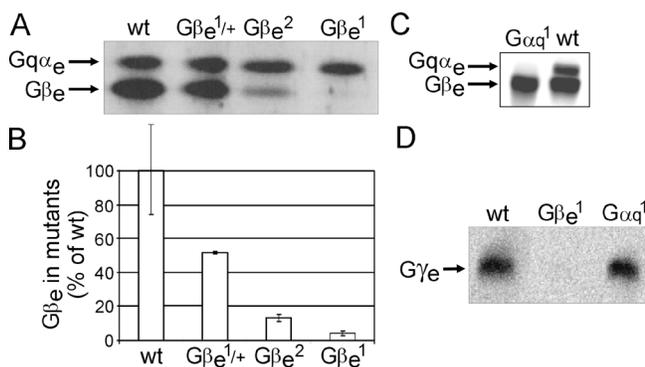


Figure 1. The levels of DGq_e subunits in Gβ_e and Gaq¹ mutants. (A) The levels of Gβ_e and Gqα_e were determined for the three different dark-adapted Gβ_e mutants (Gβ_e¹, Gβ_e², and heterozygous Gβ_e^{1/+}) using Western blot analysis. Aliquots containing equivalent protein amounts of total head homogenates were separated on a 7.5–15% gel and were visualized with a mixture of Gqα_e and Gβ_e antibodies at saturating concentrations. Each mutant has different levels of Gβ_e, whereas the level of Gqα_e remains constant. (B) Quantification of Gβ_e levels in different Gβ_e mutants. The wild-type percentage level was set as 100%. Gβ_e levels in the heterozygous Gβ_e^{1/+} mutant, the Gβ_e² mutant, and the most severe mutant, Gβ_e¹, are 50, 13, and 4%, respectively. Data represent mean values ± SEM (error bars) from seven independent experiments. (C) The levels of Gqα_e and Gβ_e in dark-adapted Gaq¹ mutant and in wild-type flies were determined using Western blot analysis. The results show that Gβ_e levels are maintained independently of Gqα_e. (D) Determination of Gγ_e levels in dark-adapted wild-type, Gβ_e¹, and Gaq¹ flies using Western blot analysis shows that the level of Gγ_e is completely dependent on Gβ_e but not on Gqα_e.

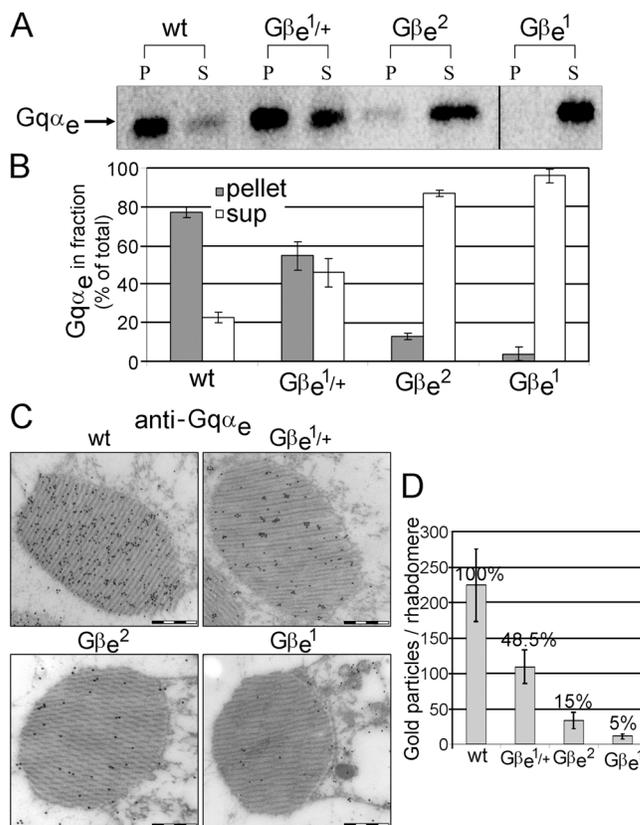


Figure 2. Gβ_e determines the membrane and rhabdomeral localization of Gqα_e. (A) Western blot analysis shows the localization of Gqα_e in the membrane (P, pellet) and in cytosol (S, supernatant) in dark-adapted Gβ_e mutants. (B) Gqα_e distribution between the membrane and cytosol of dark-adapted Gβ_e mutants and of wild-type flies is represented by the percentage of Gqα_e in each fraction (P and S) out of the total Gqα_e amount (P + S) in each mutant. Data represent mean values ± SEM from five independent experiments. (C) Immunogold EM analysis of cross sections of a single rhabdomere using Gqα_e antibodies that were applied to dark-adapted wild-type flies and Gβ_e mutants. Bars, 500 nm. (D) Number of gold particles in a cross section of a single rhabdomere. Each gold particle represents a Gqα_e molecule. Data represent mean values ± SEM (error bars) from 20 different rhabdomeres for each mutant. Wild-type percentage level was set as 100%.

and heterologous expression in tissue culture cells. Qualitatively, it is now generally accepted that plasma membrane attachment of the α subunit requires coexpression of the $\beta\gamma$ subunit complex (Degtyarev et al., 1994; Evanko et al., 2000, 2001), and, reciprocally, plasma membrane attachment of the $\beta\gamma$ subunit complex requires coexpression of the α subunit (Michaelson et al., 2002; Takida and Wedegaertner, 2003). Although a great deal has been learned from these previous studies, little is known about the localization of G protein subunits in their natural environment and how the stoichiometry of these subunits affects the level, localization, and function of G protein subunits under physiological conditions. To test the effect of various subunits on the level of one another, we have used the *Drosophila* eye-specific $G\beta$ subunit mutants ($G\beta_e$) that were described by Dolph et al. (1994) and the eye-specific $Gq\alpha$ subunit mutant ($G\alpha q^1$) that was described by Scott et al. (1995).

The hypomorph $G\beta_e$ mutants $G\beta_e^1$, $G\beta_e^2$, and the heterozygote of the most severe mutant, $G\beta_e^1/+$, express the $G\beta_e$ subunit protein at levels of 4, 13, and 50% of wild-type flies, respectively (Fig. 1, A and B). Despite the progressive decrease in the $G\beta_e$ subunit level in these mutants, the level of the α subunit was undiminished and is the same level as in wild-type flies (Fig. 1 A). Similarly, in the $G\alpha q^1$ mutant, which expresses negligible levels of the α subunit, the level of the $G\beta_e$ subunit was the same as in wild-type flies (Fig. 1 C). Although the levels of the $G\beta_e$ subunit that we found in the $G\beta_e^1$ and $G\beta_e^2$ mutants were higher than those previously reported (Dolph et al., 1994), the progressive decrease of the $G\beta_e$ subunit protein among these mutants was similar (Fig. 1 B). The eye-specific $G\gamma_e$ subunit, which forms an extremely tight complex with the $G\beta_e$ subunit, completely disappeared in the severe $G\beta_e^1$ mutant but, like $G\beta_e$, was undiminished in the $G\alpha q^1$ mutant (Fig. 1 D). Therefore, we can conclude that the $G\beta_e$ mutants are, in fact, $\beta\gamma$ mutants and that the effects observed in $G\beta_e$ mutants can be ascribed to a decrease in the level of the $\beta\gamma$ subunit dimer without effecting the level of the α subunit.

The $G\beta\gamma_e$ subunits are essential for membrane attachment and targeting of the $Gq\alpha_e$ subunit to the rhabdomere

To understand how $G\beta_e$ affects the localization of $Gq\alpha_e$, we extended our analysis to membrane attachment and targeting of the α subunit in $G\beta_e$ mutants. As shown in Fig. 2, the low levels of $\beta\gamma$ subunits in $G\beta_e$ mutants cause a progressive decrease in the fraction of the α subunit that is attached to the membrane. Quantitatively, the decrease in membrane attachment of the α subunit is proportional to the percent decrease in the level of the β subunit.

The molecules that participate in phototransduction, including the eye-specific DGq_e subunits, are confined to a specific signaling compartment (the rhabdomere). Thus, we investigated how the decreased levels of $\beta\gamma$ subunits affect the targeting of the α subunit to the signaling compartment. Using immunogold EM with antibodies against the eye-specific α subunit, we counted the gold particles in 20 cross sections of equal size from wild-type and mutant rhabdomeres. This analysis revealed that the quantity of the $Gq\alpha_e$ subunit in the rhabdomere of different mutants corresponds with the level of the α subunit that is membrane attached (Fig. 2) and indicates that the $\beta\gamma$ subunit complex controls both membrane attachment and rhabdomeral targeting of the α subunit.

The reduced light sensitivity of $G\beta_e$ mutants is caused by the mislocalization of $Gq\alpha_e$

One of the major advantages of *Drosophila* for the study of phototransduction in vivo is the ability to examine the electrophysiological response in detail and characterize the phenotype that results from a decrease in a specific phototransduction component, which is caused by mutation. Two physiological phenotypes were observed for $G\beta_e$ mutants (Dolph et al., 1994). The first phenotype was a dramatic loss of light sensitivity (reaching a decrease by two orders of magnitude in the $G\beta_e^1$ mutant), and the second phenotype was a slow termination of the light response. To address the possibility that the reduced

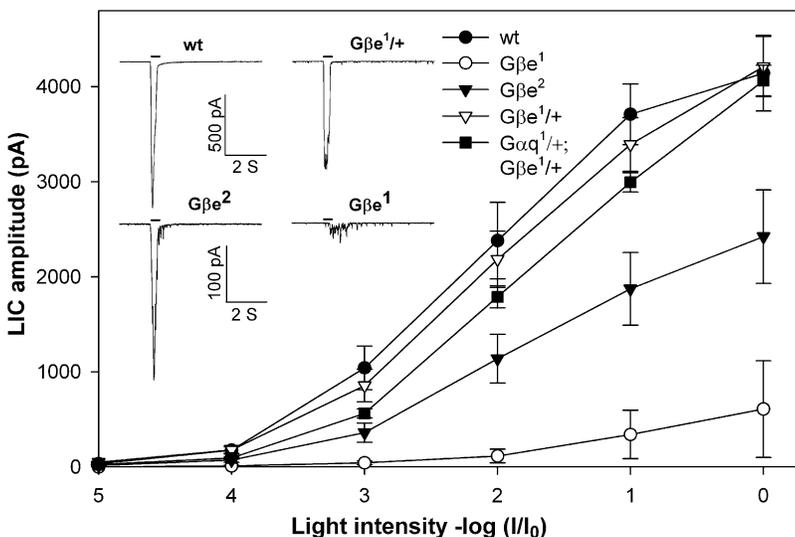


Figure 3. Sensitivity to light in wild-type flies and $G\beta_e$ mutants. Mean peak of the light-induced currents (LIC) in response to increasing intensities of orange light is plotted as a function of the light intensity. $G\beta_e$ mutants (open circle) showed a 2-log reduction in sensitivity to light, whereas $G\beta_e^2$ mutants (closed triangle) showed an ~ 0.7 log reduction in sensitivity to light. $G\beta_e^1/+$ (open triangle) and $G\alpha q^1/+; G\beta_e^1/+$ (closed square) were not significantly different from wild-type flies (closed circle). Each curve represents a mean of >10 different experiments. Error bars represent SEM. Inset shows whole cell recordings of LIC from isolated ommatidia clamped at -70 mV. The maximal orange light intensity was attenuated by 2 log units in all of the traces. Note the different scales for the top and bottom traces.

sensitivity to light in $G\beta_e$ mutants arises from a reduction in membrane-bound $Gq\alpha_e$, we reexamined the sensitivity to light in four *Drosophila* mutants with reduced levels of $G\beta_e$. Figs. 2 and 3 show a correlation between the level of membrane-bound $Gq\alpha_e$ (Fig. 2) and the sensitivity of the response to light (Fig. 3) in which low levels of membrane-bound $Gq\alpha_e$ correspond to low light sensitivity. The latter was accompanied by a modified waveform of the light-induced current (Fig. 3, inset). The fact that heterozygous $G\beta_e^{1/+}$ showed only a minor reduction in the sensitivity to light is consistent with previous results showing that 50% of $Gq\alpha_e$ is sufficient to maintain normal sensitivity to light (Scott et al., 1995). Together, these results indicate that the loss of light sensitivity is caused by the effect of $G\beta_e$ mutants on membrane attachment and targeting of the $Gq\alpha_e$ subunit to the signaling compartment (the rhabdomere). Clearly, when rhodopsin and $Gq\alpha_e$ are present in different cellular compartments, the $Gq\alpha_e$ subunit cannot transfer signals from rhodopsin to the phospholipase C enzyme.

Membrane localization of the $G\beta_e$ subunit

To examine the effect of $Gq\alpha_e$ on the localization of $G\beta_e$, we measured the distribution of $G\beta_e$ between the membrane and cytosol in wild-type and $G\alpha q^1$ mutant flies. In contrast to the light-dependent translocation of $Gq\alpha_e$ from the membrane to the cytosol (Kosloff et al., 2003), $G\beta_e$ was about equally distributed between the membrane and the cytosol under both light and dark conditions (Fig. 4, A and B). A longer period of illumination for up to 4 h did not alter the $G\beta_e$ distribution (not depicted). These results suggest that the $\beta\gamma$ complex remains partly bound to the membrane even when the α subunit is translocated to the cytosol. Indeed, it has been shown that although rhodopsin- $G\alpha$ interactions are reduced upon activation, rhodopsin- $G\beta\gamma$ interactions remain undiminished (Phillips and Cerione, 1992). Moreover, electrostatic calculations showed that upon dissociation from the $G\alpha$ subunit, the β subunit of transducin exposed a prominent patch of basic residues that enhanced the membrane affinity of the $\beta\gamma$ dimer by about an order of magnitude (Murray et al., 2001). However, it has also been shown in the rat visual system that $G\beta\gamma$ subunits translocate from the outer to the inner rod segment in response to light, albeit at a slower rate than the translocation of the α subunit (Sokolov et al., 2002).

The effect of $Gq\alpha_e$ on the membrane attachment of $G\beta_e$ was further studied using the $G\alpha q^1$ mutant. In this mutant, which has a negligible level of $Gq\alpha_e$, the $G\beta_e$ subunit is localized mainly to the cytosol (>80%; Fig. 4, A and B), suggesting that a newly synthesized $G\beta\gamma$ complex is dependent on the α subunit for membrane attachment. Failure of the $\beta\gamma$ complex to bind by itself to the plasma membrane was previously observed in transfected cells (Evanko et al., 2001; Michaelson et al., 2002; Takida and Wedegaertner, 2003) and in $G\alpha$ RNA interference of *Caenorhabditis elegans* embryos (Gotta and Ahringer, 2001). However, immunogold EM using specific antibodies against $G\beta_e$ revealed that the $\beta\gamma$ complex is targeted to the rhabdomere even in the near absence of the α subunit (Fig. 4 C) but apparently remains soluble within this compartment. This result indicates that the $\beta\gamma$ complex is targeted to the rhabdomere independently of $Gq\alpha_e$ but depends on the α subunit for

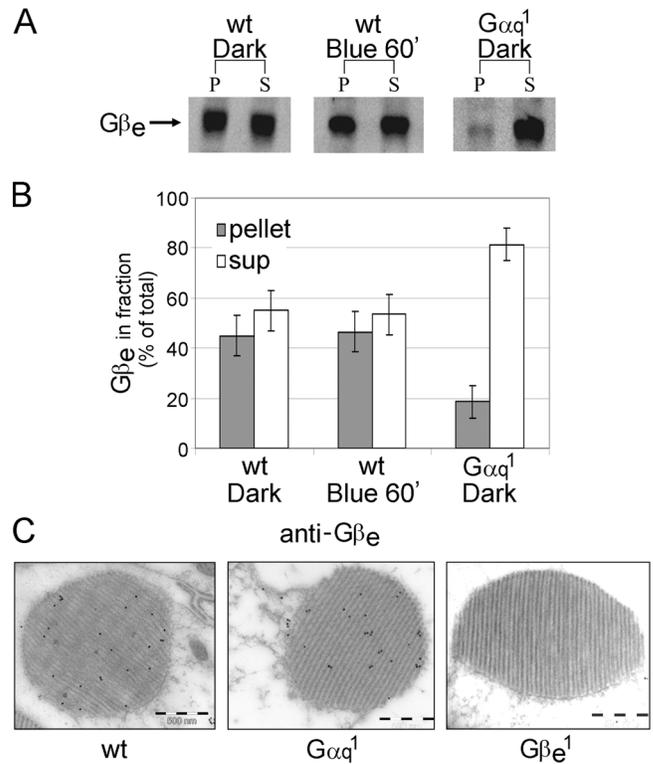


Figure 4. Membrane attachment and rhabdomeral targeting of $G\beta_e$. (A) Western blot analysis shows the localization of $G\beta_e$ in membrane (P, pellet) and in cytosol (S, supernatant) of wild-type, dark-adapted, or illuminated flies and of dark-adapted $G\alpha q^1$ flies. Illumination was with blue light for 60 min. In wild-type flies, $G\beta_e$ was about equally distributed between the membrane and the cytosol both under dark and light conditions. In $G\alpha q^1$ mutant flies, however, $G\beta_e$ failed to reach the membrane and was mostly soluble. (B) Percentage of $G\beta_e$ in fractions (P and S) out of the amount of total $G\beta_e$ (P + S) of each treatment. Data represent mean values \pm SEM (error bars) from 10 independent experiments. (C) Immunogold EM analysis of cross sections of a single rhabdomere from dark-adapted wild-type flies and $G\alpha q^1$ mutant flies using affinity-purified $G\beta_e$ antibodies revealed that $G\beta_e$ is targeted to the rhabdomere even in the near absence of $Gq\alpha_e$. $G\beta_e^{1/1}$ mutant flies were used as a control for the specificity of the antibodies. Bars, 500 nm.

tight membrane attachment. The presence of soluble $G\beta_e$ in the rhabdomere can be a result of interactions with protein partners like phosphodiesterase (Sokolov et al., 2004) and regulators of G protein signaling proteins (Snow et al., 1998). Although homologues of these proteins are present in the *Drosophila* genome, their cellular localization in *Drosophila* photoreceptors are currently unknown. The cellular localization of the Gq_e heterotrimer may be determined by the $\beta\gamma$ complex. This finding is consistent with a previous report that ectopic targeting of the $\beta\gamma$ complex to the mitochondria leads to mitochondrial localization of the $G\alpha$ subunit (Fishburn et al., 2000).

The $G\beta_e$ subunit is present in excess over the $Gq\alpha_e$ subunit

The presence of 80% of $Gq\alpha_e$ in a membrane-bound form in wild-type dark-adapted flies (Fig. 2 B, left), whereas only 50% of $G\beta_e$ is membrane bound (Fig. 4 B, left), raised the question of the stoichiometry of these two components. To determine the levels of the subunits in vivo, we performed immunoblot

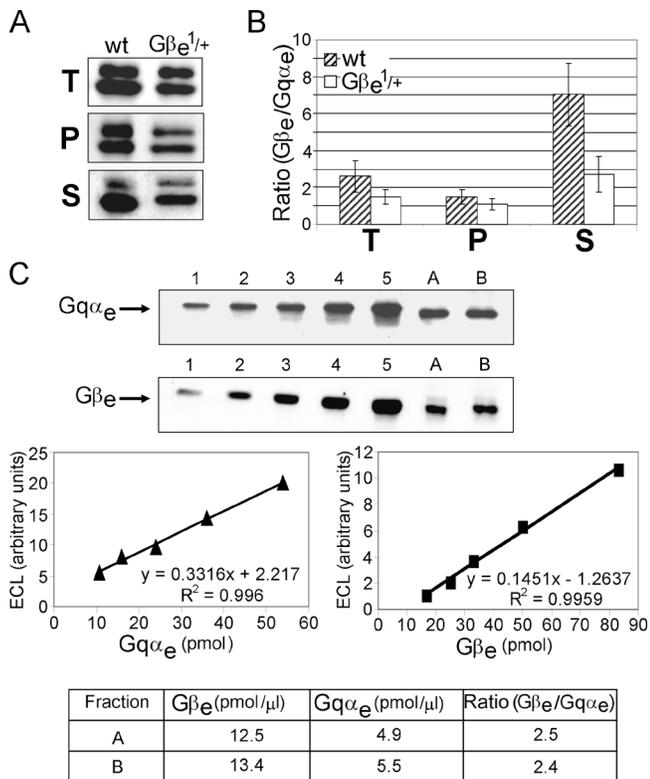


Figure 5. A physiological excess of Gβ_e over Gqα_e that is abolished in heterozygous Gβ_e^{1/+} mutant flies. (A) Western blot analysis of Gqα_e (top band) and Gβ_e (bottom band) in dark-adapted wild-type and heterozygous Gβ_e^{1/+} flies shows an excess of Gβ_e over Gqα_e in wild-type flies that is abolished in the Gβ_e^{1/+} mutant. Western blots were performed with a mixture of Gqα_e and Gβ_e antibodies. T, total amount in the cell; P, pellet (membrane); S, supernatant (cytosol). (B) The ratio between Gβ_e and Gqα_e in different fractions. Data represent mean values ± SEM (error bars) from 10 independent experiments. (C) Two samples of head homogenates (indicated as A and B) were analyzed by Western blotting along with five samples containing various amounts of recombinant *Drosophila* Gqα_e (top) and Gβ_e (bottom) standards (indicated as 1–5). Calibration curves were obtained by plotting the amount of ECL signal in each band against the amount of the recombinant protein in the standard. Because the volume of head homogenates that was applied on each gel was different in order to fit the linear range of each calibration curve, Gqα_e and Gβ_e amounts in the head homogenates were calculated for 1 μl of homogenate. Gqα_e and Gβ_e amounts in each homogenate and the ratio between them are represented in the table at the bottom. Data are representative of six independent experiments. The ratio between Gβ_e and Gqα_e was determined as 2.6 ± 0.2.

analysis with a mixture of Gqα_e- and Gβ_e-specific antibodies at a concentration five times that required for their saturation. Furthermore, two different anti-Gβ_e antibodies that were raised against two different sequences of the Gβ_e protein gave similar results (see SDS-PAGE and immunoblotting). In wild-type flies, the amount of Gβ_e was ~2.5 times higher than the amount of Gqα_e (Fig. 5, A and B). To verify the excess of Gβ_e over Gqα_e subunits in wild-type flies, which was determined by Western blotting, we calibrated the immunoblot with the use of purified recombinant *Drosophila* Gqα_e and Gβ_e proteins. We determined the concentrations of the recombinant proteins spectrophotometrically by using calculated extinction coefficients of Gqα_e = 42,350 cm⁻¹ M⁻¹ and Gβ_e = 60,000 cm⁻¹ M⁻¹ at 280 nm. This quantitative analysis of two samples of

wild-type fly head homogenate again revealed an excess of Gβ_e over Gqα_e of ~2.5 times (Fig. 5 C).

Most of the excess Gβ_e was present in the cytosol, whereas the membrane-bound fraction contained both the α and β subunits in about equal amounts (Fig. 5, A and B). Therefore, in rhabdomere membranes, all of the Gqα_e molecules, which are in close proximity to rhodopsin, may be associated with the Gβ_e subunit. This finding also indicates that in the *Drosophila* photoreceptor cells, there is a soluble pool of free Gβ_e subunit in the rhabdomere. The localization of soluble Gβ_e in the signaling organelle, the rhabdomere (Fig. 4), could be functionally important.

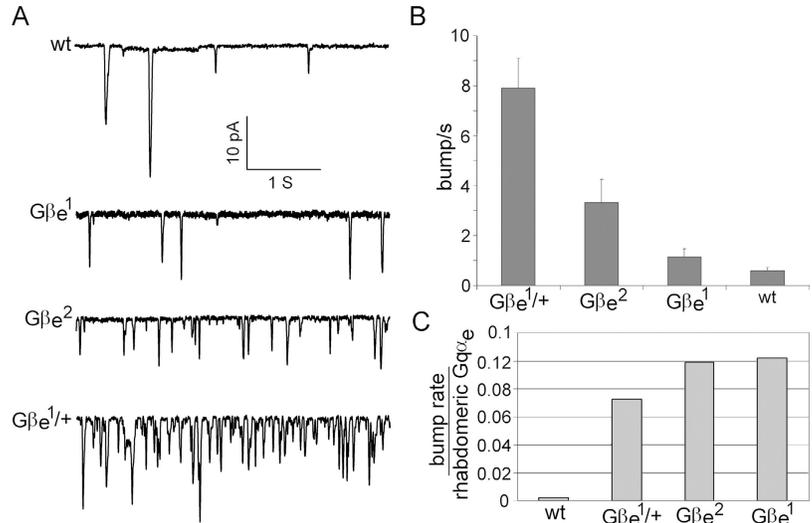
The unexpected excess of Gβ_e over Gqα_e was almost completely abolished in the Gβ_e¹ heterozygous mutant (Gβ_e^{1/+}). The ratio between Gβ_e and Gqα_e in this mutant was ~1:1. The decrease in Gβ_e levels of this mutant did not change the ratio between membrane-bound Gqα_e and Gβ_e, which remained ~1:1. In the soluble fraction, however, we found a large decrease of excess Gβ_e. Although the ratio between soluble Gβ_e and Gqα_e in wild-type flies was ~7:1, the ratio in the Gβ_e^{1/+} mutant was reduced to ~2.5:1 (Fig. 5, A and B).

Spontaneous activity of Gβ_e mutants

A new and striking phenotype of Gβ_e mutants was revealed in this study. Whole cell patch-clamp recording of dark-adapted mutant photoreceptor cells showed spontaneous, unitary, inward currents that were similar in shape to the single photon responses known as quantum bumps (Fig. 6; Henderson et al., 2000). The frequency of these spontaneous responses was different for the various Gβ_e mutants. For the Gβ_e¹ mutant, only a low frequency of spontaneous bumps was observed, which was not much different from the frequency of spontaneous bumps observed in wild-type flies. A higher frequency of spontaneous bumps was clearly noted for the Gβ_e² mutant, whereas the most dramatic increase in the frequency of spontaneous bumps was observed for the Gβ_e¹ heterozygous mutant (Gβ_e^{1/+}). The high frequency of spontaneous bumps in the heterozygous Gβ_e¹ mutant is surprising because this mutant has normal sensitivity to light in contrast to the Gβ_e¹ homozygote, which is the most severe mutant but has an almost normal frequency of spontaneous bumps (Figs. 3 and 6). This complex behavior can be explained by the decreased levels of Gqα_e observed in the signaling compartment of these mutants (Fig. 2). Indeed, when the bump frequency was normalized to the number of rhabdomeral Gqα_e, a similar bump frequency per rhabdomeral Gqα_e was observed for all of the Gβ_e mutants, whereas the wild-type bump frequency remained much lower (Fig. 6 C).

To find out whether the high frequency of spontaneous activity is caused by activation of the G protein and not by the spontaneous activation of rhodopsin, we generated a heterozygous Gβ_e^{1/+} mutant with highly decreased levels of rhodopsin. To reduce the rhodopsin level in Gβ_e^{1/+} flies, we reduced the chromophore level by raising the flies on a carotenoid-deficient medium (Minke and Kirschfeld, 1979) for three generations (Gβ_e^{1/+} Vit A⁻). The metarhodopsin potential (M potential) is a linear electrical manifestation of the level of rhodopsin in fly photoreceptors (Pak and Lidington, 1974; Minke and Kirsch-

Figure 6. **Spontaneous activation of the visual signaling cascade in $G\beta_e$ mutants.** (A) Whole cell recordings of light-induced currents from isolated ommatidia of dark-adapted $G\beta_e$ mutants and wild-type flies clamped at -70 mV. Spontaneous bumps are observed in complete darkness at different rates in the various mutants. (B) Histogram plotting the bump frequency of various mutants. Data represent mean values \pm SEM (error bars) from at least eight different experiments. The difference between the wild-type and $G\beta_e^1$ mutant is not statistically significant ($P < 0.1$). The statistics include bumps with amplitudes of >2.5 pA, which clearly exceeds the background noise. (C) The bump frequency of various $G\beta_e$ mutants and of wild-type flies was divided by the number of rhabdomeral $Gq\alpha_e$ of each mutant as determined by the immunogold labeling assay (Fig. 2 D).



field, 1980). Fig. 7 A shows the amplitude of the M potential in $G\beta_e^1/+$ flies raised on standard medium (top) compared with $G\beta_e^1/+$ flies raised on carotenoid-deficient medium (bottom). The virtually complete elimination of M potential after carotenoid deprivation clearly shows that the rhodopsin level was largely reduced in these flies. This conclusion was further supported by measuring the sensitivity to light after carotenoid deprivation, which resulted in a reduction of ~ 300 -fold in sensitivity to light without a change in the distribution of $Gq\alpha_e$ in carotenoid-deprived flies (not depicted). Fig. 7 shows that the high rate of spontaneous bumps, which is characteristic of $G\beta_e^1/+$ flies, was not significantly changed by reduced levels of rhodopsin. This indicates that the high frequency of spontaneous bumps in the $G\beta_e^1/+$ mutant does not arise from the spontaneous activation of rhodopsin in the dark.

The excess of $G\beta_e$ over $Gq\alpha_e$ that was observed in wild-type flies is almost abolished in the $G\beta_e^1/+$ heterozygous mutant (Fig. 5); this finding raised the possibility that the excess in wild-type flies prevents the spontaneous activity of $Gq\alpha_e$ observed in the $G\beta_e^1/+$ heterozygous mutant. To further test this hypothesis, we crossed the $G\beta_e^1$ mutant with the Gaq^1 mutant to generate a double mutant containing one copy of the $Gq\alpha_e$ gene and one copy of the $G\beta_e$ gene ($Gaq^1/+;G\beta_e^1/+$). The double mutant had about half the level of both $Gq\alpha_e$ and $G\beta_e$ as wild-type flies (Fig. 8 A), restoring the excess $G\beta_e$ over $Gq\alpha_e$ that was observed in wild-type flies (Fig. 8, B and C). This mutant showed almost normal sensitivity to light (Fig. 3) and no spontaneous activity in the dark (Fig. 8, D and E). This result strongly suggests that the excess of $G\beta_e$ over $Gq\alpha_e$, rather than the absolute amount of the $G\beta_e$ subunit, prevents the spontaneous activation of $Gq\alpha_e$ in *Drosophila* photoreceptor cells.

Discussion

The decreased light sensitivity of *Drosophila* $G\beta_e$ mutants

When $G\beta_e$ mutants were first isolated (Dolph et al., 1994), it was reported that these mutations caused a dramatic decrease in the sensitivity to light, which was ascribed to participation

of the β subunit in G protein–rhodopsin coupling. Our finding that the decrease in $G\beta_e$ in $G\beta_e$ mutants is accompanied by a proportional decrease in $Gq\alpha_e$ in the rhabdomeral compartment does not support the previously claimed catalytic effect of the β subunit on light sensitivity (Dolph et al., 1994). Rather, we conclude that the decrease in light sensitivity of these mutants is caused by the presence of rhodopsin and the major fraction of the G protein α subunit in two different cellular compartments. Clearly, when these two components are present in different cellular locations, the photo-excited rhodopsin is unable to catalyze the exchange of GDP that is bound to the $Gq\alpha_e$ for free GTP, and the transduction process is prematurely terminated. The mechanism that underlies the decreased sensitivity to light in $G\beta_e$ mutants, therefore, is a structural change in the localization of the $Gq\alpha_e$ subunit.

We also examined how a decrease in the α subunit of the Gaq^1 mutant influences membrane attachment and targeting of the $\beta\gamma$ subunits to the rhabdomere. In this case, the $\beta\gamma$ dimer is soluble and not membrane attached but is still targeted to the rhabdomere. The presence of $\beta\gamma$ in the rhabdomeral cytosol may be physiologically important for preventing spontaneous activity because the $\beta\gamma$ subunits are in close proximity to the membrane-bound signaling molecules.

We have previously shown that the eye-specific $Gq\alpha_e$ subunit translocates from rhabdomeral membranes to the cytosol in response to illumination (Kosloff et al., 2003). $Gq\alpha_e$ behaves like many other $G\alpha$ subunits, which demonstrate activity-dependent translocation from the membrane to the cytosol (for review see Resh, 1999; Chen and Manning, 2001; Smotrys and Linder, 2004). The *Drosophila* eye-specific $\beta\gamma$ dimer behaves differently from the $Gq\alpha_e$ subunit, as it does not show any significant change in its distribution even after prolonged illumination (Fig. 4). A possible reason for this result might be an interaction between the γ subunit of the $\beta\gamma$ dimer and the photoactivated rhodopsin. Such an interaction has been reported for the transducin γ subunit and the active form of vertebrate rhodopsin (Kisselev and Downs, 2003). Both vertebrate and invertebrate photoreceptor cells contain high concentrations of rhodopsin, and even a weak interaction could be significant as a result of

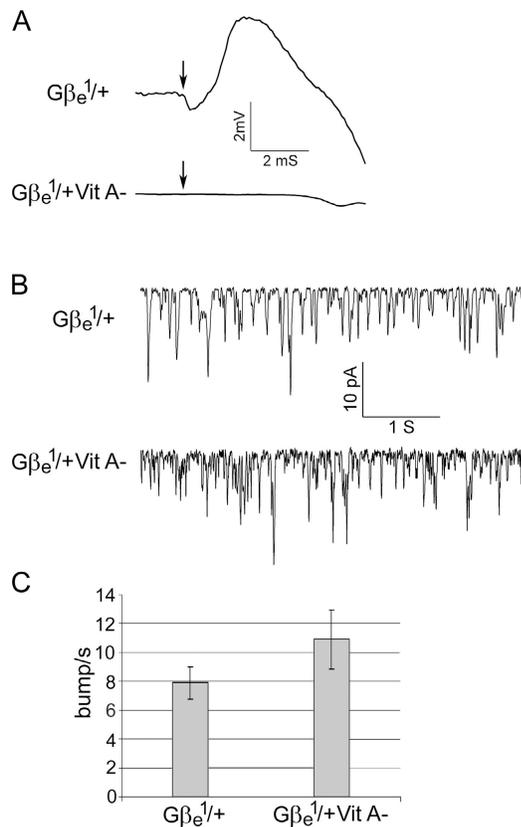


Figure 7. Rhodopsin is not essential for spontaneous bump production in the $G\beta_e^{1/+}$ mutant. (A) ERG recordings of $G\beta_e^{1/+}$ and $G\beta_e^{1/+}$ Vit A- responses to a white flash, indicated by arrows. $G\beta_e^{1/+}$ flies that were raised on standard medium displayed M potential similar to that of wild-type flies, whereas the M potential of $G\beta_e^{1/+}$ Vit A- was abolished. Traces shown are means of 20 consecutive experiments. (B) Whole cell recordings of light-induced currents from isolated ommatidia of dark-adapted $G\beta_e^{1/+}$ mutants and $G\beta_e^{1/+}$ Vit A- flies clamped at -70 mV. A similar frequency of spontaneous bump was observed in flies reared under both conditions. (C) Histogram plotting the bump frequency of $G\beta_e^{1/+}$ and $G\beta_e^{1/+}$ Vit A- flies in the dark. Data represents mean values \pm SEM (error bars) from eight different experiments. The difference between $G\beta_e^{1/+}$ and $G\beta_e^{1/+}$ Vit A- is not statistically significant ($P < 0.1$). The statistics include only bumps with an amplitude of >2.5 pA, which clearly exceeds the background noise.

mass action. It should be noted, however, that studies in rat retina detected light-dependent movement of both the α and $\beta\gamma$ subunits from the rod outer to inner segment, although the $\beta\gamma$ subunits moved more slowly than the α subunit, suggesting that it might be caused by an interaction of the $\beta\gamma$ complex with phosducin (Sokolov et al., 2002, 2004). The different behavior of $G\beta\gamma$ subunits in vertebrate and *Drosophila* might be caused by the difference in stability of the active rhodopsin in these two systems. Whereas vertebrate rhodopsin undergoes bleaching and inactivation, the activated rhodopsin of *Drosophila* is stable for hours (Minke and Selinger, 1996).

Spontaneous, dark photoreceptor activity in $G\beta_e$ mutants

A functional hallmark of visual photoreceptors is utmost sensitivity of the capacity for single photon detection. This sensitivity is achieved by very high concentrations of the photoreceptor

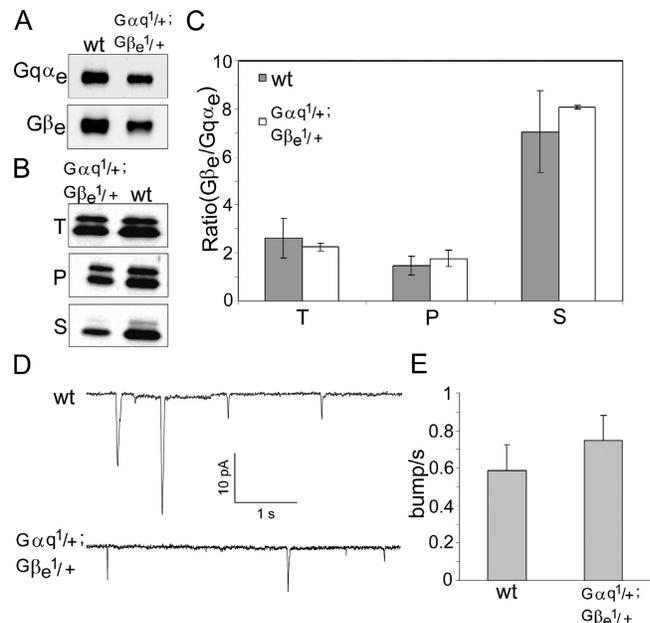


Figure 8. The $G\beta_e$ excess over $Gq\alpha_e$ is restored in a $G\alpha q^{1/+};G\beta_e^{1/+}$ double mutant. This mutant has almost no spontaneous activity in the dark. (A) $G\alpha q^{1/+};G\beta_e^{1/+}$ double mutants were generated by the crossing of $G\beta_e^{1/+}$ mutants with $G\alpha q^{1/+}$ mutants. Western blot analysis shows $G\beta_e$ and $Gq\alpha_e$ levels in dark-adapted wild type and in the $G\alpha q^{1/+};G\beta_e^{1/+}$ double mutant. Quantification of the ECL signal shows that the $Gq\alpha_e$ level was reduced to $\sim 50\%$ and that the $G\beta_e$ level was reduced to $\sim 60\%$ of wild-type values. The experiment was repeated three times. (B) Western blot analysis of $Gq\alpha_e$ (top band) and $G\beta_e$ (bottom band) in dark-adapted wild-type and $G\alpha q^{1/+};G\beta_e^{1/+}$ mutants shows that the excess of $G\beta_e$ over $Gq\alpha_e$ is restored in this mutant. Western blots were performed with a mixture of $Gq\alpha_e$ and $G\beta_e$ antibodies. T, total amount in the cell; P, pellet (membrane); S, supernatant (cytosol). (C) The ratio between $G\beta_e$ and $Gq\alpha_e$ in different fractions of the $G\alpha q^{1/+};G\beta_e^{1/+}$ double mutant is very similar to that of wild-type flies. Data represent mean values \pm SEM from five different experiments. Data from wild-type flies are the same as in Fig. 5 B and are shown here only for comparison. (D) Whole cell recordings of light-induced currents from isolated ommatidia of dark-adapted $G\alpha q^{1/+};G\beta_e^{1/+}$ double mutants and of wild-type flies clamped at -70 mV. Data from wild-type flies are the same as in Fig. 6 A and are shown here only for comparison. (E) Histogram plotting the bump frequency of the $G\alpha q^{1/+};G\beta_e^{1/+}$ double mutant compared with that of wild-type flies. Data represent mean values \pm SEM (error bars) from at least eight different experiments. The difference between wild-type and $G\alpha q^{1/+};G\beta_e^{1/+}$ double mutants is not statistically significant ($P < 0.1$). The statistics include bumps with amplitudes of >2.5 pA, which clearly exceeds the background noise. Data of wild-type flies are the same as in Fig. 6 B and are shown here only for comparison (note the different bars of Figs. 6 B and 8 E).

rhodopsin and its target G protein as well as by the large amplification that is generated during the phototransduction process. High sensitivity also depends on an exceedingly low spontaneous activity (low, dark noise) that sets the limit on the absolute sensitivity of this signaling system. Rhodopsin is the only G protein-coupled receptor that has covalently linked 11 cis-retinal that behaves like a "quasi" antagonist in the dark, preventing spontaneous activity. The visual G protein, however, needs special mechanisms to prevent spontaneous activation, but these mechanisms remain unknown.

The $G\beta\gamma$ subunits are known to bind to $G\alpha$ -GDP switch regions, thereby stabilizing the binding of GDP and suppressing spontaneous receptor-independent activation (Itoh and Gilman,

1991; Lambright et al., 1996; Sondek et al., 1996; Preininger and Hamm, 2004). To find out whether this interaction is relevant to the *Drosophila* eye-specific Gq heterotrimer, whose three-dimensional structure has not been determined, we have constructed a homology model of the DGq_e heterotrimer ($\alpha\beta\gamma$) based on the crystal structure of transducin (unpublished data). It appears from the model that the G $\beta\gamma$ _e subunit complex directly contacts the switch I and switch II space regions of Gq α _e as was previously reported for other G proteins. Therefore, it is conceivable that the *Drosophila* G $\beta\gamma$ _e complex prevents the spontaneous activation of Gq α _e by binding to Gq α _e switch regions. It should be pointed out, however, that the physiological consequences of this effect in vivo have not been described previously. Furthermore, the mechanism that suppresses the spontaneous activity of G proteins under physiological conditions is unknown. In this study, we report (Fig. 6) the observation of a high frequency of spontaneous activity in the heterozygous G β _e^{1/+} mutant in which the level of the β subunit was decreased to 50% of its level in wild-type flies. Surprisingly, a further decrease in the level of G β _e in the G β _e² and G β _e¹ mutants did not increase the frequency of spontaneous activity but rather decreased the frequency. This is easily seen in the most severe mutant (G β _e¹), which has only 4% of G β _e, as the spontaneous activity is not much different from the low frequency in wild-type flies. This is probably the reason why the increase in spontaneous activity was not detected in the initial characterization of G β _e mutants (Dolph et al., 1994). These results indicate that the relationship between the level of G β _e and the spontaneous activity is not straightforward. We suggest that the observed spontaneous activity of β mutant photoreceptor cells is regulated by two opposite effects of the $\beta\gamma$ dimer. On the one hand, the decrease in $\beta\gamma$ levels leaves some G α -GDP unassociated with $\beta\gamma$, and this free G α -GDP undergoes spontaneous exchange of the bound GDP for free GTP, leading to spontaneous activity. On the other hand, the decreased level of $\beta\gamma$ leads to a proportional decrease of G α in the signaling compartment, resulting in a diminished ability to activate the phototransduction process. To test this notion, we normalized the observed rate of spontaneous activity to the number of Gq α _e subunits in the rhabdomeres of G β _e mutants that lack excess G β _e over Gq α _e. We found (Fig. 6 C) similar frequencies of normalized spontaneous activity for all of the G β _e mutants, which is consistent with a role of excess $\beta\gamma$ over Gq α _e in suppressing spontaneous activity.

The presence of excess G β _e over Gq α _e in the *Drosophila* photoreceptor cell

One of the unexpected and novel findings of this study is the presence of the *Drosophila* eye-specific G β _e subunit in ~2.5-fold excess over the Gq α _e subunit. Because the levels of α and β subunit proteins are maintained independently of one another, unequal levels of these subunits are mechanistically possible. Our calibration curves using purified recombinant G β _e and Gq α _e proteins (Fig. 5 C) verified the excess of G β _e over Gq α _e subunits, which was determined by immunoblot analysis with a mixture of Gq α _e and G β _e antibodies (Fig. 5 A). Furthermore, we have shown that as long as the two antibodies are

maintained at saturating concentrations and determinations are performed in the same gel, levels of the α and β subunits are obtained that nicely fit the expected results from gene dosage effects (Fig. 5 A). Furthermore, according to the “two-signal model” for membrane attachment of peripheral membrane proteins, one expects to find equal amounts of membrane-bound Gq α _e and G β _e subunits. In accord with this notion, although we found about twofold excess of total G β _e over Gq α _e, an analysis of these subunits in the membrane-bound fraction gave a ratio of 1:1.

In the heterozygous G β _e^{1/+} mutant, in which there is a reduction of 50% in the level of the β subunit, yielding a β/α ratio of ~1, we found a dramatic increase in the spontaneous activity of photoreceptor cells (Figs. 5 and 6). The critical role of the excess of G β _e over Gq α _e was revealed in the Gq α ^{1/+}; G β _e^{1/+} double heterozygous mutant, in which the rate of spontaneous activity was dramatically reduced by restoring the excess of G β _e over Gq α _e. This indicates that the excess of G β _e, rather than its absolute amount, is important to maintain a low frequency of spontaneous activity. Furthermore, this mutant rules out the possibility that the spontaneous activity we observed was caused by side effects of the G β _e mutation. Altogether, this is the first demonstration of the strategy of excess $\beta\gamma$ over the α subunit in vivo for the suppression of spontaneous activity at the G protein level.

Two possible mechanisms can explain how the excess of G β _e over Gq α _e prevents spontaneous activity. One mechanism could be through participation of the soluble pool of rhabdomeral G β _e in accelerating the hydrolysis of Gq α _e-GTP if spontaneous exchange occurs. This mechanism is currently under investigation. The second mechanism could be through the stabilization of Gq α _e-GDP, thus preventing the exchange of bound GDP for free GTP. In an insightful, theoretical paper dealing with the spontaneous activity of G proteins by using thermodynamic model simulations, it was found that the concentration of β equal to that of α is barely sufficient to suppress spontaneous activity, whereas a twofold excess of $\beta\gamma$ over the α subunit produces a large decrease in spontaneous activity (Onaran et al., 1993). Altogether, our in vivo studies point to the importance of $\beta\gamma$ subunits as principle modulators of spontaneous activity and to the relevance of this strategy in vivo.

Materials and methods

Fly stocks

Drosophila of the following strains were used: wild-type, Oregon-R w (obtained from W.L. Pak, Purdue University, West Lafayette, IN); Gq α ¹, a severe hypomorph for Gq α _e (obtained from C.S. Zuker, University of California, San Diego, San Diego, CA; Scott et al., 1995); G β _e¹, a severe hypomorph mutant of eye-specific G β _e; and G β _e², a less severe hypomorph mutant of eye-specific G β _e (obtained from C.S. Zuker; Dolph et al., 1994).

Assay of light-dependent G β _e localization

Assay for the light-dependent localization of G β _e was performed as described previously (Kosloff et al., 2003). In short, dark-adapted flies were subjected to illumination with activating blue light (18-W white light lamp with a 1-mm-thick wide band filter [Schott BG 28; Bes Optics] 12 cm away from the flies) for various durations at 22°C. Termination was performed by moving the flies to 4°C in the dark and promptly separating the fly heads. 10 flies were used for each time point.

Preparation of *Drosophila* head homogenate and fractionation

Heads were separated from 10 flies that were dark adapted overnight (except in Fig. 4) and homogenized in 1 ml isotonic homogenization buffer (20 mM Tris, pH 7.5, 120 mM KCl, 0.1 mM MgCl₂, 0.1 mM PMSF, and 5 mM β-mercaptoethanol). Homogenate was either directly precipitated with 5% TCA or subjected to fractionation. Membranes and cytosol fractions were separated by centrifugation (15,800 g for 15 min at 4°C). The pellet was washed and centrifuged again, and the supernatants were combined. Ultracentrifugation at 150,000 g for 30 min did not change the distribution of α and β subunits between the fractions. The proteins were precipitated by 5% TCA, ran on SDS-PAGE, and subjected to quantification as described in SDS-PAGE and immunoblotting.

Preparation of recombinant *Drosophila* Gqα₆ and Gβ₆ proteins

cDNA clones of Gqα₆ and Gβ₆ genes were obtained from the Medical Research Council UK gene service. Gqα₆ cDNA was amplified and cloned into pQE-80 vector (QIAGEN) that contained an NH₂-terminal 6× His tag and was expressed in Rosetta bacterial cells (Novagen). The recombinant (His)₆-Gqα₆ protein was then purified on a Ni-Sepharose column (GE Healthcare) and eluted with a 20–250-nM imidazole gradient using fast protein liquid chromatography Akta explorer (GE Healthcare).

Gβ₆ cDNA was amplified and cloned into pHis-parallel 1 (pET22) vector (obtained from P. Sheffield, University of Virginia, Charlottesville, VA) that contained an NH₂-terminal 6× His tag and was expressed in HMS174 bacterial cells (Novagen). Purified recombinant (His)₆-Gβ₆ was extracted from inclusion bodies by applying 6 M guanidine HCl on a bacterial membrane extract that had been washed three times with 1% Triton X-100. Both proteins were ~95% pure as determined by SDS-PAGE and Coomassie blue staining. Recombinant protein concentrations were determined spectrophotometrically by using a calculated molar extinction coefficient of 42,350 for Gqα₆ and 60,000 for Gβ₆ at 280 nm.

SDS-PAGE and immunoblotting

Equal protein amounts that were determined by Bradford assay were loaded on the specified gel. For detection of the α or β subunits of DGq₆, a 10% SDS-PAGE was used. To detect both subunits (α and β) on the same gel, proteins were separated on a gradient 7.5–15% SDS-PAGE. For the detection of Gγ₆, a 20% SDS-PAGE with 4 M urea was used. The urea was needed for separation of the γ subunit from the β subunit. Subsequent to SDS-PAGE separation, proteins were subjected to Western blot analysis using the specified antibodies.

Two different anti-Gβ₆ polyclonal antibodies were made in rabbit as described previously (Palczewski et al., 1993). One antibody was made against a peptide from the COOH terminus of the protein (residues 333–346), and the other was made against a peptide from the NH₂ terminus (residues 3–13).

For Gqα₆ detection, we used anti-Gqα₆ polyclonal antibodies that were previously made by us (Kosloff et al., 2003), and for Gγ₆ detection, rabbit polyclonal antibodies that were directed against the Calliphora Gγ₆ protein were used (obtained from A. Huber, University of Karlsruhe, Karlsruhe, Germany; Schulz et al., 1999).

To determine the ratio between Gqα₆ and Gβ₆ subunits, we performed Western blot analysis using a mixture of anti-Gqα₆ and anti-Gβ₆ each at a 1:1,000 dilution, which is five times higher than their saturating concentration. To rule out the possibility that the Gβ₆ excess we observed is caused by the antibodies, we repeated these experiments with the two different Gβ₆ antibodies and obtained the same results. To further ensure that the Gβ₆ excess over Gqα₆ was not a result of the antibody concentrations, we repeated this procedure with a higher concentration of anti-Gqα₆ or with a twofold dilution (1:2,000) of anti-Gβ₆. In all of these cases, an excess of Gβ₆ over Gqα₆ was observed.

Relative protein amounts on the same gel were determined by quantification of the ECL signal by using the Plus Gel system (LAS-1000; Fuji).

Immunogold EM

Immunogold EM was performed as described previously (Kosloff et al., 2003). All sections were made from flies that were dark adapted overnight. Sections were incubated with either Gqα₆ antibodies (dilution of 1:80) or Gβ₆ affinity-purified antibodies (dilution of 1:20). Gβ₆ antibodies were affinity purified by using Affi-Gel 10 gel (Bio-Rad Laboratories) according to the manufacturer's instructions for anhydrous coupling followed by elution with glycine-HCl, pH 2.5. The secondary antibody used was goat anti-rabbit conjugated to 18 nm of gold particles.

Sections were observed and photographed with a transmission electron microscope (Technai-12; Philips) equipped with a CCD camera

(MegaView II; Soft Imaging System) and were visualized with analySIS 3.0 image processing software (Soft Imaging System).

Electroretinogram (ERG) and M potential

ERG recordings were performed on intact flies as described previously (Peretz et al., 1994). Orange light (OG-590 Schott edge filter; Bes Optics) from a Xenon high pressure lamp (operating at 50 W; model LPS 220; Photon Technology International) was delivered to the compound eye by an optic fiber and was attenuated by natural density filters. The maximal luminous intensity at the eye surface was 12.5 mW/cm². M potential recordings were performed as described previously (Minke and Kirschfeld, 1980). In brief, an adapting light of maximal intensity 20-s blue light (Schott BG-28) from the Xenon high pressure lamp was delivered 1 min before each white test stimulus (70 joules of photographic flash light).

Whole cell recording

Dissociated ommatidia were prepared from newly eclosed white-eyed adult flies (<1 h after eclosion; Hardie, 1991) that were maintained in a 12-h dark/12-h light cycle and kept in the dark 24 h before the experiment. Whole cell patch-clamp recordings were performed as previously described (Hardie and Minke, 1992). Signals were amplified with a patch-clamp amplifier (Axopatch 200B; Axon Instruments, Inc.), sampled at 2,000 Hz, and filtered below 1,000 Hz. The bath solution contained 120 mM NaCl, 5 mM KCl, 10 mM N-Tris buffer, pH 7.15, 4 mM MgSO₄, and 1.5 mM CaCl₂. The pipette solution contained 120 mM K gluconate, 2 mM MgSO₄, 10 mM N-Tris buffer, pH 7.15, 4 mM MgATP, 0.4 mM Na₂GTP, and 1 mM NAD⁺.

Transillumination of the halogen light source (100 W) was used as previously described (Peretz et al., 1994). The orange stimulating light (Schott OG-590) was applied via a condenser lens (Carl Zeiss Microimaging, Inc.) and was attenuated by neutral density filters.

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