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

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*Drosophila 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 ($\alpha\beta\gamma$) G protein revealed that wild-type *Drosophila* flies have about a twofold excess of $\text{G}\beta$ over $\text{G}\alpha$ subunits of the visual Gq protein. Studies of $\text{G}\beta_e$ mutants in which the excess of $\text{G}\beta$ was genetically eliminated showed dramatic dark, spontaneous activity of the photoreceptor cells, whereas concurrent genetic reduction of the $\text{G}\alpha$ subunit, which restored the excess of $\text{G}\beta$, abolished this effect. These results indicate that an excess of $\text{G}\beta$ over $\text{G}\alpha$ 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 $\alpha$ subunit. G proteins that are charged with guanosine diphosphate (GDP) are in the inactive state, where the $\alpha$ 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; Smotrys and Linder, 2004). All G protein $\alpha$ subunits (with the exception of transducin) are palmitoylated, and some are additionally modified by myristoylation.

The $\alpha$ subunits of G$\gamma$/D,$\gamma$-$\delta$,$\gamma$-$\delta$, including the *Drosophila melanogaster* eye–specific G$\alpha_q$, as well as G$\alpha_s$, G$\gamma$-$\delta$, and G$\gamma$-$\delta$-$\alpha$ are modified only by palmitoylation. The corresponding $\beta\gamma$ subunits undergo isoprenylation of a cysteine residue at the so-called CAAX box of the $\gamma$ subunit (for review see Wedegaertner, 1998; Resh, 1999; Chen and Manning, 2001). Plasma membrane attachment of the $\alpha$ subunits Gs$\alpha$ and Gq$\alpha$ 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 $\gamma$ subunit, are poorly targeted to the plasma membrane and require coexpression of the $\alpha$ 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 $\alpha$ and $\beta\gamma$ subunits of the heterotrimeric G protein). This procedure is bound to cause distortion of the original stoichiometry of $\alpha$ 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.
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 (Gqα, Scott et al., 1995), which, in turn, activates the phospholipase C enzyme effector (Devary et al., 1987). Upon activation, the eye-specific Gq subunit (Gqαe) dissociates from the eye-specific Gβ dimer (Gβγe) 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 Gβ1, hypomorph mutants) that the Gβ 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 Gqα1, we found that the Gβ dimer is dependent on the α subunit for membrane attachment but not for targeting to the rhabdomere, suggesting a role for the Gβ dimer in targeting the heterotrimer to the photoreceptor signaling compartment (the rhabdomere). An analysis of the protein levels of Gqαe and Gβ2 subunits revealed a surprising twofold excess of the Gβ2 subunit over the Gqαe 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 Gqαe and, thereby, restored the excess of Gβ2 over Gqαe 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 Gqαe and Gβ2 subunits in Drosophila photoreceptors are maintained independently of one another. The α subunit of the heterotrimeric G protein and the tightly associated complex of Gβ 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](image1.png)

**Figure 1.** The levels of Gqα, subunits in Gβ1, and Gqα1 mutants. (A) The levels of Gβ2, and Gqαe were determined for the three different dark-adapted Gβ1 mutants (Gβ21, Gβ22, and heterozygous Gβ2+/+) 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 Gqαe, and Gβ2 antibodies at saturating concentrations. Each mutant has different levels of Gβ2, whereas the level of Gqαe remains constant. (B) Quantification of Gβ2 levels in different Gβ2 mutants. The wild-type percentage level was set as 100%. Gβ2 levels in the heterozygous Gβ2+/+ mutant, the Gβ2−/− mutant, and the most severe mutant, Gβ2−/−, are 50, 13, and 4%, respectively. Data represent mean values ± SEM from five independent experiments. (C) The levels of Gqαe and Gβ2 in dark-adapted Gqα1 mutant and in wild-type flies were determined using Western blot analysis. The results show that Gβ2 levels are maintained independently of Gqαe. (D) Determination of Gγ, levels in dark-adapted wild-type, Gβ2, and Gqα1 flies using Western blot analysis shows that the level of Gγ, is completely dependent on Gβ2, but not on Gqαe.

![Figure 2](image2.png)

**Figure 2.** Gβ2 determines the membrane and rhabdomeral localization of Gqαe. (A) Western blot analysis shows the localization of Gqαe in the membrane (P, pellet) and in cytosol (S, supernatant) in dark-adapted Gβ1 mutants. (B) Gqαe distribution between the membrane and cytosol of dark-adapted Gβ2 mutants and of wild-type flies is represented by the percentage of Gqαe in each fraction (P and S) out of the total Gqαe 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 Gqαe antibodies that were applied to dark-adapted wild-type flies and Gβ2 mutants. Bars, 500 nm. (D) Number of gold particles in a cross section of a single rhabdomere. Each gold particle represents a Gqαe molecule. Data represent mean values ± SEM 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 βγ subunit complex (Degtyarev et al., 1994; Evanko et al., 2000, 2001), and, reciprocally, plasma membrane attachment of the βγ 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 Gβ subunit mutants (Gβ) that were described by Dolph et al. (1994) and the eye-specific Gqα subunit mutant (Gaq1) that was described by Scott et al. (1995).

The hypomorph Gβ, mutants Gβ1, Gβ2, and the heterozygote of the most severe mutant, Gβ1/+ , express the Gβ 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 Gβ subunit level in these mutants, the level of the α subunit was unimpaired and is the same level as in wild-type flies (Fig. 1 A). Similarly, in the Gaq1 mutant, which expresses negligible levels of the α subunit, the level of the Gβα subunit was the same as in wild-type flies (Fig. 1 C). Although the levels of the Gβ subunit that we found in the Gβ1 and Gβ2 mutants were higher than those previously reported (Dolph et al., 1994), the progressive decrease of the Gβ subunit protein among these mutants was similar (Fig. 1 B). The eye-specific Gγ subunit, which forms an extremely tight complex with the Gβ subunit, completely disappeared in the severe Gβ1 mutant but, like Gβα, was unimpaired in the Gaq1 mutant (Fig. 1 D). Therefore, we can conclude that the Gβ mutants are, in fact, βγ mutants and that the effects observed in Gβ mutants can be ascribed to a decrease in the level of the Gβ subunit dimer without effecting the level of the α subunit.

The Gβγα subunits are essential for membrane attachment and targeting of the Gqα subunit to the rhabdomere

To understand how Gβγα affects the localization of Gqα, we extended our analysis to membrane attachment and targeting of the α subunit in Gβγα mutants. As shown in Fig. 2, the low levels of βγ subunits in Gβγα 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 DGqα subunits, are confined to a specific signaling compartment (the rhabdomere). Thus, we investigated how the decreased levels of βγ 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 Gqα 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 βγ subunit complex controls both membrane attachment and rhabdomeral targeting of the α subunit.

The reduced light sensitivity of Gβα mutants is caused by the mislocalization of Gqα

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 Gβα 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 Gβ1 mutant), and the second phenotype was a slow termination of the light response. To address the possibility that the reduced
sensitivity to light in Gββ, mutants arises from a reduction in membrane-bound Gqα, we reexamined the sensitivity to light in four Drosophila mutants with reduced levels of Gββ. Figs. 2 and 3 show a correlation between the level of membrane-bound Gqα (Fig. 2) and the sensitivity of the response to light (Fig. 3) in which low levels of membrane-bound Gqα 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 Gββ1/+ showed only a minor reduction in the sensitivity to light is consistent with previous results showing that 50% of Gqα 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 Gββ mutants on membrane attachment and targeting of the Gqα subunit to the signaling compartment (the rhabdomere). Clearly, when rhodopsin and Gqα are present in different cellular compartments, the Gqα subunit cannot transfer signals from rhodopsin to the phospholipase C enzyme.

Membrane localization of the Gβε subunit
To examine the effect of Gqα on the localization of Gβε, we measured the distribution of Gβε between the membrane and cytosol in wild-type and Gqα1 mutant flies. In contrast to the light-dependent translocation of Gqα from the membrane to the cytosol (Kosloff et al., 2003), Gβε 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 Gβε distribution (not depicted). These results suggest that the βγ 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–αγ interactions are reduced upon activation, rhodopsin–Gβγ interactions remain undiminished (Phillips and Cerione, 1992). Moreover, electrostatic calculations showed that upon dissociation from the αγ subunit, the β subunit of transducin exposed a prominent patch of basic residues that enhanced the membrane affinity of the βγ dimer by about an order of magnitude (Murray et al., 2001). However, it has also been shown in the rat visual system that Gβγ 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 Gqα on the membrane attachment of Gβε was further studied using the Gqα1 mutant. In this mutant, which has a negligible level of Gqα, the Gβε subunit is localized mainly to the cytosol (>80%; Fig. 4, A and B), suggesting that a newly synthesized Gβγ complex is dependent on the α subunit for membrane attachment. Failure of the βγ complex to bind by itself to the plasma membrane was previously observed in transfected cells (Evaniko et al., 2001; Michaelson et al., 2002; Takida and Wedegaertner, 2003) and in Gα RNA interference of Caenorhabditis elegans embryos (Gotta and Ahringer, 2001). However, immunogold EM using specific antibodies against Gβε revealed that the βγ 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 βγ complex is targeted to the rhabdomere independently of Gqα, but depends on the α subunit for tight membrane attachment. The presence of soluble Gβε in the rhabdomere can be a result of interactions with protein partners like phosducin (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 GqGα heterotrimer may be determined by the βγ complex. This finding is consistent with a previous report that ectopic targeting of the βγ complex to the mitochondria leads to mitochondrial localization of the Gqα subunit (Fishburn et al., 2000).

The Gβε subunit is present in excess over the Gqα subunit
The presence of 80% of Gqα in a membrane-bound form in wild-type dark-adapted flies (Fig. 2 B, left), whereas only 50% of Gβε 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
Furthermore, two different anti-Gα concentration five times that required for their saturation.

We determined the concentrations of the recombinant proteins spectrophotometrically by using calculated extinction coefficients of Gα = 42,350 cm⁻¹ M⁻¹ and Gβ = 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 over Gα of ~2.5 times (Fig. 5 C).

Most of the excess Gβ 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 rhodobemere membranes, all of the Gα molecules, which are in close proximity to rhodopsin, may be associated with the Gβ subunit. This finding also indicates that in the Drosophila photoreceptor cells, there is a soluble pool of free Gβ, subunit in the rhodobemere. The localization of soluble Gβ, in the signaling organelle, the rhodobemere (Fig. 4), could be functionally important.

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

**Spontaneous activity of Gβ, mutants**
A new and striking phenotype of Gβ, 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, mutants. For the Gβ, 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, heterozygous mutant (Gβ,+/+), whereas the most dramatic increase in the frequency of spontaneous bumps was observed for the Gβ, heterozygous mutant (Gβ,+/+). The high frequency of spontaneous bumps in the heterozygous Gβ, mutant is surprising because this mutant has normal sensitivity to light in contrast to the Gβ, 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 Gα observed in the signaling compartment of these mutants (Fig. 2). Indeed, when the bump frequency was normalized to the number of rhodobemeral Gα, a similar bump frequency per rhodobemeral Gα was observed for all of the Gβ, 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,/+ mutant with highly decreased levels of rhodopsin. To reduce the rhodopsin level in Gβ,/+ flies, we reduced the chromophore level by raising the flies on a carotenoid-deficient medium (Minke and Kirschfeld, 1979) for three generations (Gβ,/+ 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 Gβɛ mutants. (A) Whole cell recordings of light-induced currents from isolated ommatidia of dark-adapted Gβɛ 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 ± SEM (error bars) from at least eight different experiments. The difference between the wild-type and Gβɛ1 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 Gβɛ mutants and of wild-type flies was divided by the number of rhabdomeral Gqαɛ of each mutant as determined by the immunogold labeling assay (Fig. 2 D).

The excess of Gβɛ over Gqαɛ that was observed in wild-type flies is almost abolished in the Gβɛ1/+ heterozygous mutant (Fig. 5); this finding raised the possibility that the excess in wild-type flies prevents the spontaneous activation of Gqαɛ observed in the Gβɛ1/+ heterozygous mutant. To further test this hypothesis, we crossed the Gβɛ1 mutant with the Gaq1 mutant to generate a double mutant containing one copy of the Gqαɛ gene and one copy of the Gβɛ gene (Gaqa1+/Gβɛ1/−). The double mutant had about half the level of both Gqαɛ and Gβɛ as wild-type flies (Fig. 8 A), restoring the excess Gβɛ over Gqαɛ 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 Gβɛ over Gqαɛ, rather than the absolute amount of the Gβɛ subunit, prevents the spontaneous activation of Gqαɛ in Drosophila photoreceptor cells.

Discussion

The decreased light sensitivity of Drosophila Gβɛ mutants

When Gβɛ 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 Gβɛ in Gβɛ mutants is accompanied by a proportional decrease in Gqαɛ 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 Gqαɛ for free GTP, and the transduction process is prematurely terminated. The mechanism that underlies the decreased sensitivity to light in Gβɛ mutants, therefore, is a structural change in the localization of the Gqαɛ subunit.

We also examined how a decrease in the α subunit of the Gaq1 mutant influences membrane attachment and targeting of the βγ subunits to the rhabdomere. In this case, the βγ dimer is soluble and not membrane attached but is still targeted to the rhabdomere. The presence of βγ in the rhabdomeral cytosol may be physiologically important for preventing spontaneous activity because the βγ subunits are in close proximity to the membrane-bound signaling molecules.

We have previously shown that the eye-specific Gqαɛ subunit translocates from rhabdomeral membranes to the cytosol in response to illumination (Koslof et al., 2003). Gqαɛ behaves like many other Gα subunits, which demonstrate activity-dependent translocation from the membrane to the cytosol (for review see Resh, 1999; Chen and Manning, 2001; Smotrycz and Linder, 2004). The Drosophila eye–specific βγ dimer behaves differently from the Gqαɛ 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 βγ 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
mass action. It should be noted, however, that studies in rat retina detected light-dependent movement of both the α and βγ subunits from the rod outer to inner segment, although the βγ subunits moved more slowly than the α subunit, suggesting that it might be caused by an interaction of the βγ complex with phosphducin (Sokolov et al., 2002, 2004). The different behavior of Gβγ 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 Gβα 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 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 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 Gβγ subunits are known to bind to Gα-GDP switch regions, thereby stabilizing the binding of GDP and suppressing spontaneous receptor-independent activation (Itoh and Gilman,
The presence of excess Gβe over Gqαe in the Drosophila photoreceptor cell

One of the unexpected and novel findings of this study is the presence of the Drosophila eye-specific Gβe subunit in ~2.5-fold excess over the Gqαe 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 Gβe and Gqαe proteins (Fig. 5 C) verified the excess of Gβe over Gqαe subunits, which was determined by immunoblot analysis with a mixture of Gqαe and Gβe 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 Gqαe and Gβe subunits. In accord with this notion, although we found about twofold excess of total Gβe over Gqαe, an analysis of these subunits in the membrane-bound fraction gave a ratio of 1:1.

In the heterozygous Gβe+/+ 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 Gβe over Gqαe was revealed in the Gaq1/+; Gβe+/+ double heterozygous mutant, in which the rate of spontaneous activity was dramatically reduced by restoring the excess of Gβe over Gqαe. This indicates that the excess of Gβe, 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 Gβe mutation. Altogether, this is the first demonstration of the strategy of excess βγ 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 Gβe over Gqαe prevents spontaneous activity. One mechanism could be through participation of the soluble pool of rhodomeral Gβe in accelerating the hydrolysis of Gqαe-GTP if spontaneous exchange occurs. This mechanism is currently under investigation. The second mechanism could be through the stabilization of Gqαe-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 βγ 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 βγ 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 C.S. Zuker, University of California, San Diego, San Diego, CA; Scott et al., 1995); Gβe1, a severe hypomorph for Gqαe (obtained from C.S. Zuker; Dolph et al., 1994), a severe hypomorph mutant of eye-specific Gβe; and Gβe2, a less severe hypomorph mutant of eye-specific Gβe (obtained from C.S. Zuker; Dolph et al., 1994).

Assay of light-dependent Gβe localization

Assay for the light-dependent localization of Gβe, 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 cytosolic 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 Gαq and Gβγ proteins
cDNA clones of Gαq and Gβγ genes were obtained from the Medical Research Council UK gene service. Gαq cDNA was amplified and cloned into pQE-80 vector (QIAGEN) that contained an NH₂-terminal 6x His tag and was expressed in Rosetta bacterial cells [Novagen]. The recombinant (His)₅₋Gαq protein was then purified on a Ni-Sepharose column (GE Healthcare) eluted and subjected to a 20–250 nm imidazole gradient using fast protein liquid chromatography Aktaprobe (GE Healthcare).

Gβγ cDNA was amplified and cloned into pET22 (PET22) vector (obtained from P. Sheffield, University of Virginia, Charlottesville, VA) that contained an NH₂-terminal 6x His tag and was expressed in HMS174 bacterial cells [Novagen]. The recombinant (His)₅₋Gβγ 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 Aktaprobe (GE Healthcare). Gβγ protein concentrations were determined spectrophotometrically by using a calculated molar extinction coefficient of 42,350 for Gαq, and 60,000 for Gβγ 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 DQα, a 10% SDS-PAGE was used. To detect both subunits (α and β) on the same protein was separated on a gradient 7.5–15% SDS-PAGE. For the detection of Gβγ, a 20% SDS-PAGE with 4 M urea was used. The urea was added 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-Gβγ polyclonal antibodies were made in rabbit as described previously (Polczewski 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 Gαq detection, we used anti-Gαq, polyclonal antibodies that were previously made by us (Kosloff et al., 2003), and for Gαq detection, rabbit polyclonal antibodies that were directed against the COOH terminus of the protein were used (obtained from A. Huber, University of Karlsruhe, Karlsruhe, Germany; Schulz et al., 1999).

To determine the ratio between Gαq, and Gβγ subunits, we performed Western blot analysis using a mixture of anti-Gαq, and anti-Gβγ, each at 1:1,000 dilution, which is five times higher than their saturating concentration. To rule out the possibility that the Gβγ excess we observed is caused by the antibodies, we repeated these experiments with the two different Gβγ antibodies and obtained the same results. To further ensure that the Gβγ excess over Gαq was not a result of the antibody concentrations, we repeated this procedure with a higher concentration of anti-Gαq, or with a twofold dilution [1:2,000] of anti-Gβγ. In all of these cases, an excess of Gβγ over Gαq 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 incubated with either Gαq or Gβγ antibodies (dilution of 1:80) or Gβγ affinity-purified antibodies (dilution of 1:20). Gβγ antibodies were affinity purified by using Affi-Gel 10 gel (Bio-Rad Laboratories) according to the manufacturer’s instructions for anhydroxy coupling followed by elution with glycine-HCl, pH 2.5. The secondary antibody 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 filters; Bausch & Lomb) was observed and photographed at Xenon high pressure lamp (operating at 50 W; model JPS 220; Photon Technology International) was delivered to the compound eye by an optic fiber and was attenuated by neutral 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 20–5 blue light (Schott BG-28) from the Xenon high pressure lamp was delivered 1 min before each white test stimulus (70° 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 NaTris 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 NaTris buffer, pH 7.15, 4 mM MgATP, 0.4 mM NaGTP, and 1 mM NaNO₃. Transillumination of the halogen light source (100 W) was used as described previously (Peretz et al., 1994). The orange stimulating light (Schott OG-590) was applied via a condenser lens (Carl Zeiss Microimag, Inc.) and was attenuated by neutral density filters.

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