Molecular Basis of Amplification in *Drosophila* Phototransduction: Roles for G Protein, Phospholipase C, and Diacylglycerol Kinase

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Summary

In *Drosophila* photoreceptors, the amplification responsible for generating quantum bumps in response to photoisomerization of single rhodopsin molecules has been thought to be mediated downstream of phospholipase C (PLC), since bump amplitudes were reportedly unaffected in mutants with greatly reduced levels of either G protein or PLC. We now find that quantum bumps in such mutants are reduced ∼3- to 5-fold but are restored to near wild-type values by mutations in the *rdgA* gene encoding diacylglycerol kinase (DGK) and also by depleting intracellular ATP. The results demonstrate that amplification requires activation of multiple G protein and PLC molecules, identify DGK as a key enzyme regulating amplification, and implicate diacylglycerol as a messenger of excitation in *Drosophila* phototransduction.

Introduction

Many photoreceptors generate discrete responses to effective absorptions of single photons, known as quantum bumps. In *Drosophila*, these represent simultaneous activation of about 15 light-sensitive channels generating an inward current of ∼10 pA (Henderson et al., 2000). The response is remarkable for its speed, with latencies as short as 20 ms and bump halfwidths of ∼20 ms. These kinetics are about 100 times faster than in toad rods recorded at similar temperatures (Baylor et al., 1979), about 10 times faster than mammalian rods recorded at 37°C (Baylor et al., 1984), and in general, fly photoreceptors are considered to have the fastest known G protein-coupled signaling cascades (reviewed by Hardie and Raghu, 2001). The events leading to quantum bump generation in *Drosophila* have been inferred from a variety of physiological, biochemical, and genetic evidence (reviewed by Hardie and Raghu, 2001; Minke and Hardie, 2000; Montell, 1999). Photoisomerized rhodopsin activates a heterotrimeric G protein (Gq class) releasing the α subunit, which in turn activates phospholipase C (PLCβ4 isoform) encoded by the *norpA* gene (Bloomquist et al., 1988). By a still unknown mechanism, activation of PLC leads to the opening of at least two classes of Ca^{2+} permeable channels, TRP and TRPL (Hardie and Minke, 1992; Niemeyer et al., 1996; Reuss et al., 1997). These are the prototypical members of

the TRP ion channel superfamily responsible for a wide variety of Ca^{2+} influx pathways throughout the body (reviewed by Clapham et al., 2001; Montell, 2001). Recent evidence suggests that the *Drosophila* channels, as well as some vertebrate TRP homologs, may be activated by lipid second messengers rather than by $InsP_3$ (Chyb et al., 1999; Raghu et al., 2000a), though this is still controversial (Agam et al., 2000; Cook and Minke, 1999). Candidates include diacylglycerol (DAG), polyunsaturated fatty acids (PUFAs) which are DAG metabolites, or a reduction in phosphatidyl inositol 4,5 bisphosphate (PIP_2). Following activation of the first channel(s), Ca^{2+} influx mediates rapid positive and negative feedback, which is required for both amplification and rapid termination of the quantum bump (Henderson et al., 2000). Most, if not all, elements of the phototransduction cascade are located in the ∼30,000 tightly packed microvilli, each only 60 nm in diameter and 1–2 μm long, which together form the light-guiding rhabdomere. Quantitative Western analysis suggests that there are about 25 TRP channels per microvillus (Huber et al., 1996), which corresponds closely to the number of channels activated during the quantum bump (Henderson et al., 2000). It is therefore plausible that quantum bump generation is restricted to a single microvillus, representing activation of all or most of the available channels.

In contrast to the situation in vertebrate rods, recent evidence has led to the proposal that all amplification in this system is mediated downstream of PLC (Scott et al., 1995; Scott and Zuker, 1998). The main evidence for this view is that quantum bump amplitude is reported to be unaffected in hypomorphic mutants of G protein (*Gaq*) and PLC (*norpA*) where protein levels are reduced to levels (<1%) such that there may often be no more than a single G protein or PLC molecule in each microvillus (Cook et al., 2000; Pak et al., 1976; Scott et al., 1995; Scott and Zuker, 1998). This led to the conclusion that levels of G protein do not actively contribute to the gain of the single photon response and that the G protein must act as a "molecular switch," triggering bump generation (Scott et al., 1995).

In the present study, we were led to question this view by the observation of spontaneous events in the dark in WT flies. Although our data suggested they were due to spontaneous activation of G proteins, they were much smaller than quantum bumps, seemingly inconsistent with single G proteins triggering full-sized bumps. We therefore systematically reinvestigated quantum bumps in both *Gaq* and *norpA* hypomorphs and found that, contrary to previous reports, bump amplitudes were much reduced, suggesting that there is substantial amplification upstream of PLC. We resolved the discrepancy with earlier studies by showing that bump amplitude in both *Gaq* and *norpA* mutants could be increased to near WT levels by omitting ATP from the whole-cell recording pipette. Finally, we identified DAG kinase as the critical ATP-dependent factor, strongly supporting the proposal that DAG (or its downstream metabolites)

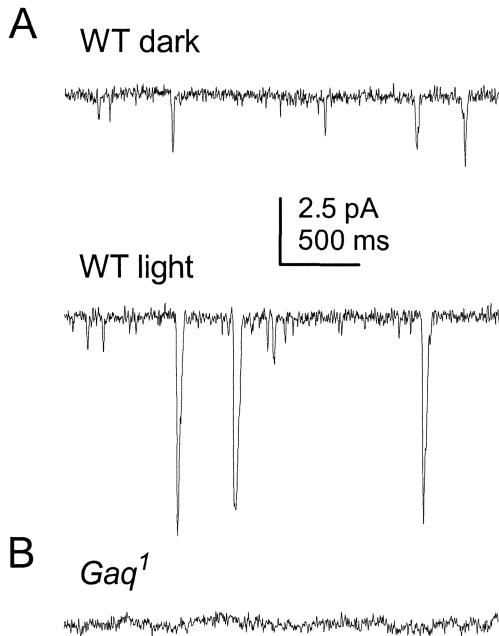[3]Correspondence: rch14@hermes.cam.ac.uk

A WT dark

2.5 pA
500 ms

WT light

B Gaq^1

Figure 1. Dark Noise

(A) Whole-cell recordings from WT photoreceptor clamped at −70mV. Small spontaneous events, ~2 pA in amplitude, are observed in complete darkness (above) and are clearly distinguishable from the three large quantum bumps induced by continuous dim light in the same cell (below).

(B) No spontaneous events are seen in $G\alpha q^1$ under similar recording conditions.

is the excitatory messenger responsible for channel activation.

Results

Spontaneous Dark Noise

As previously described, quantum bumps recorded by whole-cell voltage clamp in wild-type (WT) *Drosophila* photoreceptors under physiological conditions are ~10 pA in amplitude (Henderson et al., 2000). Although spontaneous quantum bumps (presumably representing thermal isomerizations of rhodopsin) are generated at rates of less than one per minute in complete darkness, we detected much smaller spontaneous events ~2 pA in amplitude, occurring at rates of up to about two to three per second (Figure 1). By analogy with similar "dark noise" in vertebrate photoreceptors (Rieke and Baylor, 1996), we suspected that these events might reflect spontaneous activation of one or other of the intermediate elements of the phototransduction cascade, namely, G protein or PLC. To test this, we recorded from $G\alpha q^1$, which is a severe hypomorphic mutant of the Gq α subunit, in which G protein levels are reduced 100-fold (corresponding to about one molecule per microvillus), while levels of other key transduction proteins, including rhodopsin, G_β, PLC (Scott et al., 1995), and TRP (P.R., unpublished data), are unaffected. Indeed, such events were absent, or their frequency was greatly reduced (<1 min^{-1}) in Gaq^1 photoreceptors, suggesting that they reflect spontaneous activation of Gq and subsequent downstream activation of PLC (Figure 1).

Quantum Bumps in Gaq^1 Mutants

It was previously reported that quantum bump amplitude and kinetics in Gaq^1 were indistinguishable from WT, leading to the conclusion that activation of a single G protein is sufficient to generate a full-sized bump and that levels of Gq do not actively contribute to the gain of the single photon response (Scott et al., 1995). Since this appears to be inconsistent with our interpretation of the much smaller "dark events" reflecting spontaneous activation of G proteins, we reinvestigated light responses in Gaq^1.

As reported by Scott et al. (1995), photoreceptors in Gaq^1 mutants show a drastic (~1000-fold) reduction in sensitivity to light (Figure 2), and responses to brief flashes decay abnormally slowly, returning to baseline with a time constant of ~100 ms (cf. ~20 ms in WT). This macroscopic response phenotype results from a massive reduction in quantum efficiency (Q.E. = percentage of absorbed photons eliciting a bump) along with an increase in bump latency; the latter presumably because of the greater average diffusional distance between an activated rhodopsin and G protein when there are only one or at most a few G proteins in each microvillus (Scott et al., 1995; Scott and Zuker, 1998). In contrast to these previous studies, however, we found that quantum bump size and kinetics were also profoundly affected in Gaq^1. As described in detail below, this striking discrepancy can be attributed to different recording conditions. Under our conditions, bump amplitudes were on average ~3- to 4-fold smaller (2–3 pA in Gaq^1 cf. ~10 pA in WT), bump duration was reduced (halfwidth 13 ms cf. 20 ms in WT), and the integral current, probably the most informative measure of relative amplification, was reduced ~5- to 6-fold (~50 pA.ms cf. 250–300 pA.ms in WT) (Figures 2 and 3). All these values are indistinguishable from the properties of the spontaneous dark events in WT flies (Figure 3E). However, the bump amplitude distributions of the light-induced bumps in Gaq^1 showed a long skewed tail with indications of multiple peaks at ~2, 4, and 6 pA, while the spontaneous dark events had a more symmetrical distribution with a single peak around 2 pA (Figure 2E). This can be understood if some photon absorptions in Gaq^1 result in activation of more than one G protein.

To provide evidence that these small events in Gaq^1 still represented single effective photon absorptions, we tested whether their frequency rose linearly with intensity. For small responses, the effective number of quantum bumps can be obtained by integrating under the entire response and dividing by the average single bump current integral recorded in the same cell. Since rhodopsin levels are normal in Gaq^1, intensity can be expressed in terms of the number of effective rhodopsin photoisomerizations using calibrations from bump counts in WT flies. Assuming a value of 30,000 microvilli (Gaq^1 photoreceptors have WT morphology), this can be further converted into the number of photoisomerizations per microvillus. The number of effective quantum bumps rose linearly with intensity up to about five to ten photoisomerizations per microvillus (Figure 2). Interestingly, responses then clearly saturated at levels corresponding to about 500 to 1000 quantum bumps—i.e., in only about 1/50 of the available microvilli, even though the density of residual G protein in Gaq^1 should be about one per microvillus. This suggests that activation of a
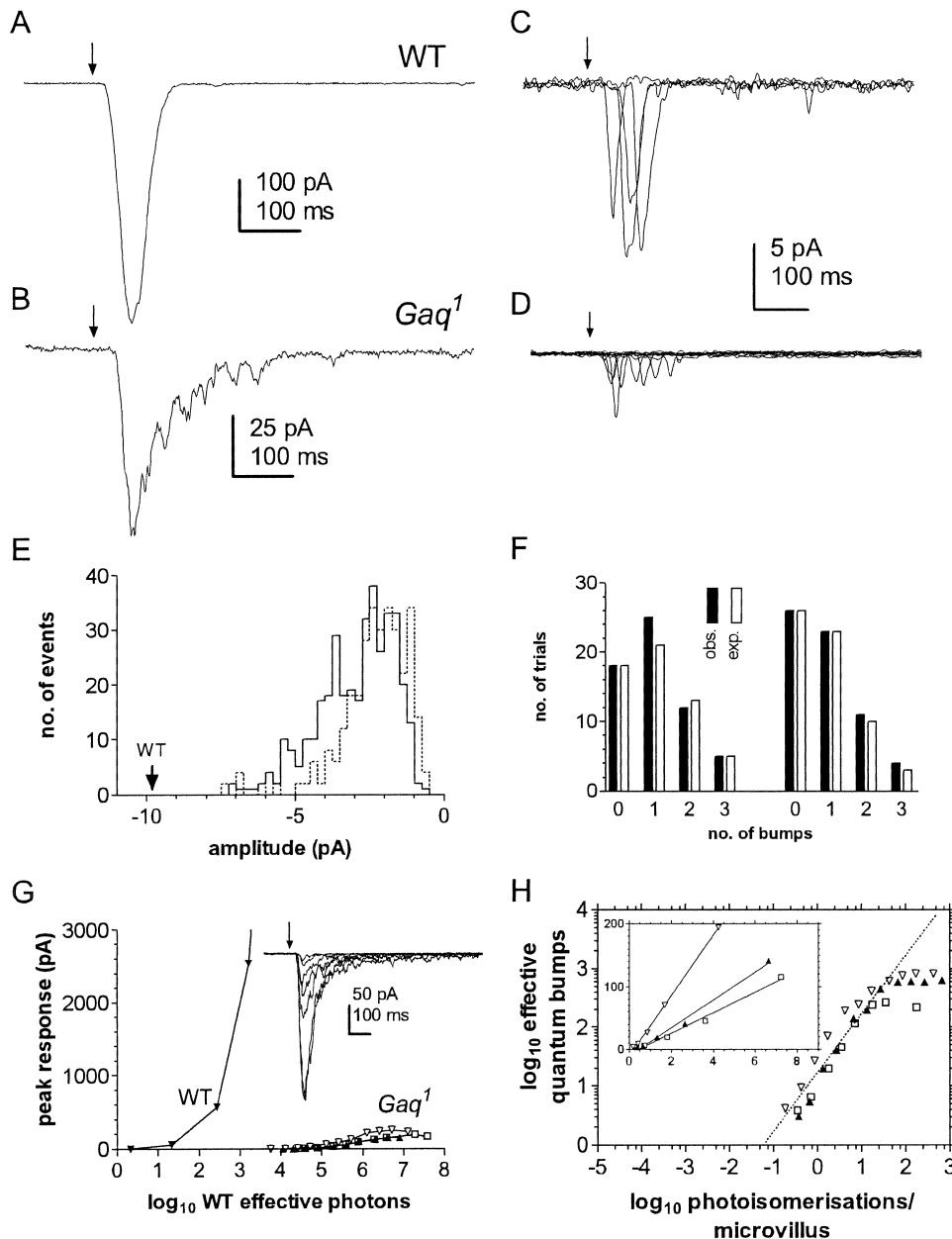
Figure 2. Responses in *Gaq^1*

(A) Responses to brief flashes (5 ms at arrow) in (A) WT (∼100 effective photons) and (B) Gαq^1 (24,000 photons). (C and D) Quantum bumps elicited by brief flashes in WT and Gαq^1 (four to six superimposed traces, selected for single bumps). (E) Histograms of bump amplitudes in *Gaq^1* (solid line, 372 bumps from six cells) and dark events in WT photoreceptors (dotted line, 160 events, three cells; scaled for comparison); arrow indicates the mean amplitude of WT quantum bumps. (F) Frequency distribution of number of trials resulting in failures (0), 1, 2, or 3 quantum bumps in two *Gaq^1* cells, following stimulation by repeated dim flashes eliciting on average 1.2 (left) or 0.89 (right) bumps. The observed (obs.) frequencies conform closely (p > 0.85; χ^2 test) to the expected Poisson prediction (see Equation 1). (G) Inset: responses in *Gaq^1* to flashes of increasing intensity; peak responses from this and two other cells are plotted against $\log_{10}$ WT effective photons with data from a typical WT photoreceptor for comparison. (H) Data from the same three *Gaq^1* cells on double $\log_{10}$ plot expressed in terms of effective quantum bumps against the number of effective photoisomerizations per microvillus (see text for further details). Dotted line is a slope of 1 (direct proportionality); inset shows the data from the initial slopes on a linear plot.

single G protein, at least in Gαq^1, has only a low probability of generating a quantum bump.

In addition, in several cases we determined the statistics of quantum bump occurrence in response to repeated flashes producing on average only one quantum bump. If these are generated by single photons, albeit with greatly reduced Q.E., their occurrence should con-

form to Poisson statistics, since photons are absorbed randomly. Specifically, the number of events (*n*) elicited by a flash eliciting on average *m* events is predicted by the Poisson distribution

$$P_n = e^{-m} \times m^n/n! \qquad (1)$$
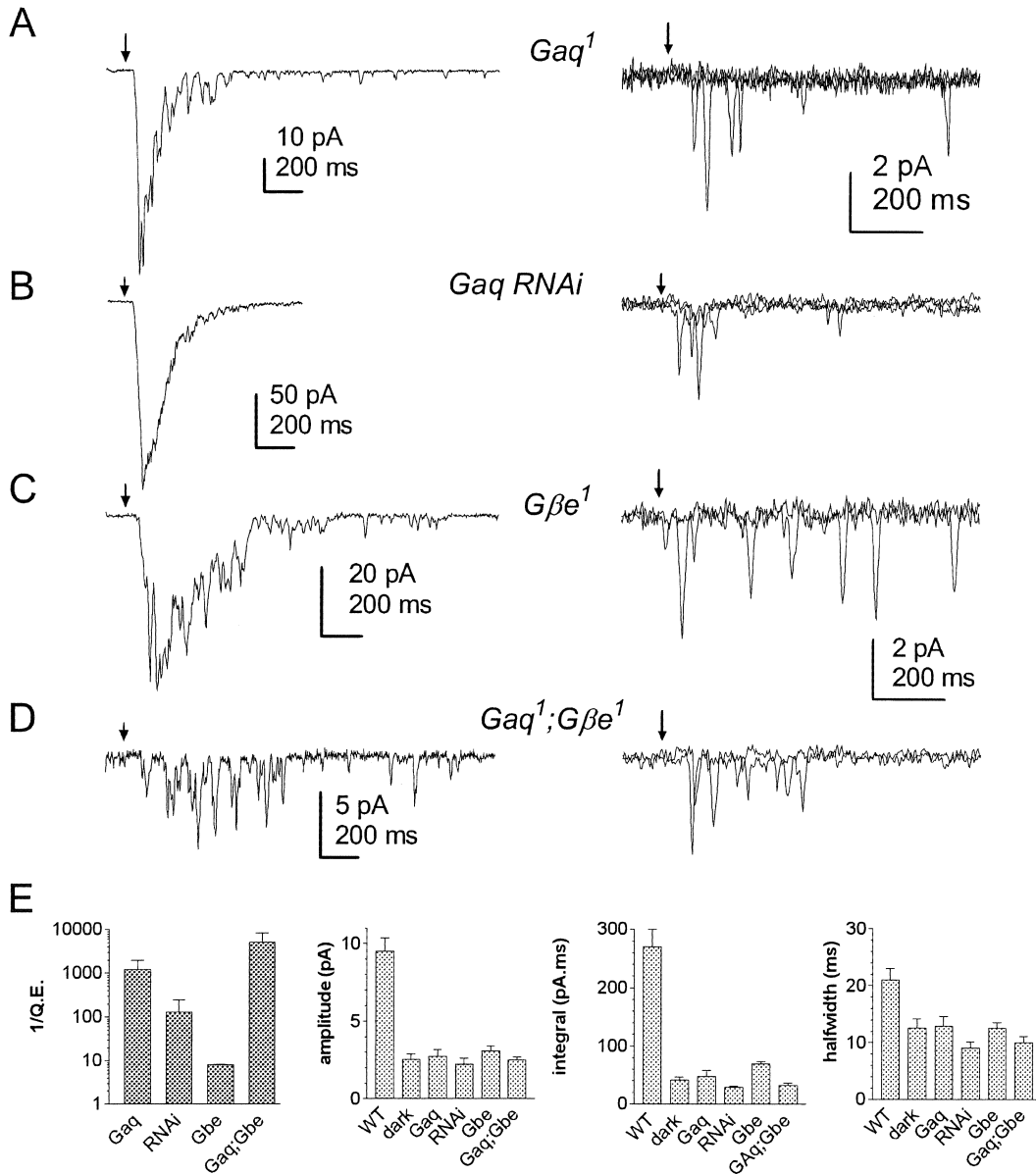
where P_n is the probability of generating *n* bumps. As

A

Gaq[1]

10 pA
200 ms

2 pA
200 ms

B

Gaq RNAi

50 pA
200 ms

C

Gβe[1]

20 pA
200 ms

2 pA
200 ms

D

Gaq[1];Gβe[1]

5 pA
200 ms

E

Figure 3. Comparison of Macroscopic Responses and Quantum Bumps in Gαq Mutants

(A–D) Left: responses to brief flashes in Gαq[1] (10[5] photons), Gaq RNA$_i$ (10[5] photons); Gβe[1] (~2000 photons), and Gαq[1];Gβe[1] double mutants (2 × 10^{6} photons). Right: superimposed traces showing quantum bumps elicited by brief flashes (arrows).

(E) Bar graphs showing reciprocal of quantum efficiency relative to WT (1/Q.E.); bump amplitude, bump current integral, and bump halfwidth for WT bumps, spontaneous dark events in WT (dark), and bumps in Gαq[1], Gaq RNA$_i$; Gβe[1], and Gαq[1];Gβe[1] double mutants. Mean ± SD (across cells), n = 3–9 cells.

shown in Figure 2, the occurrence of failures (no bumps), single, double, and treble bumps closely followed the predicted distribution, consistent with their generation by single photon absorptions.

Reduced G Protein Levels in Flies Expressing Wild-Type Gq α

The striking reduction in bump amplitude in Gaq[1] supports our interpretation that the dark events represent spontaneous activation of G proteins but appears to invalidate the conclusion that levels of G protein do not contribute to amplification (Scott et al., 1995). However,

not only do Gαq[1] mutants have reduced levels of G protein, but the residual protein is also defective, lacking 3 amino acids (residues 154–156) (Scott et al., 1995). Although these do not reside in domains believed to interact with receptor (rhodopsin) or effector (PLC), we wondered whether the reduced bump amplitudes might be a consequence of defective protein rather than lower protein levels. We used two approaches to exclude this possibility.

First, we investigated flies in which Gq α subunit levels had been reduced by RNA interference (RNAi), using a double-stranded RNA construct under control of a ret-

ina-specific promoter (GMR). This RNAi construct was previously reported to be as effective as the *Gaq¹* mutation in reducing sensitivity when measured by electroretinogram (Kalidas and Smith, 2002). We confirmed that the *Gaq¹* phenotype was effectively mimicked in whole-cell recordings from *Gaq* RNAi flies, although Q.E. was only about 100 times reduced compared to WT (cf. ∼1000 times in *Gaq¹*). Most importantly for the present purpose, bump amplitude was reduced to values as small as in *Gaq¹*, although the residual Gq α subunit is now WT (Figure 3).

Second, we investigated a mutant of the Gβ subunit (*Gβe¹*) reported to produce <1% of the WT protein levels (Dolph et al., 1994). *Gβe¹* mutants also have a much-reduced sensitivity, interpreted as being due to reduced levels of heterotrimeric G protein (Dolph et al., 1994). Again, despite a WT α subunit, quantum bump amplitudes in *Gβe¹* mutants were much smaller than in WT flies but were similar to those recorded in *Gaq¹* (Figure 3). Q.E. in *Gβe¹* was only ∼10-fold reduced compared to WT; this may indicate that the free Gq α subunits, which are presumably present in excess in *Gβe¹*, can still be activated by rhodopsin, albeit with a lower efficiency. An additional feature of *Gβe¹* was the occurrence of spontaneous dark events, indistinguishable from those seen in response to illumination and similar to the small dark events seen WT photoreceptors. It seems likely that they represent spontaneous activation of free Gq α subunits.

Since levels of Gβ are reported to be normal in *Gaq¹* (Scott et al., 1995) we also considered the possibility that the reduction in bump amplitude resulted from a mismatch in subunit stoichiometry, e.g., there might be an inhibitory effect of free Gβγ subunits, which are presumably present in excess in *Gaq¹* and *Gaq* RNAi flies. The small bumps measured in *Gβe¹* already argue against this possibility, but as an additional control, we generated *Gaq¹;Gβe¹* double mutants in which α and β subunits should be approximately matched in abundance. Bump amplitudes were again indistinguishable from *Gaq¹* (Figure 3); the only difference noted from *Gaq¹* was a further ∼10-fold reduction in sensitivity, as would be expected if some of the residual Gq α protein was inactive due to failure to assemble with Gβγ.

We conclude that the reduced quantum bump amplitude in the various Gq mutant backgrounds can be attributed to the reduced protein levels, strongly suggesting that activation of multiple G proteins is required for and directly contributes to the gain of phototransduction in *Drosophila*.

Quantum Bump Amplitude Is Reduced in *norpA* Hypomorphs

A single Gq α subunit is believed to activate just one effector (PLC) molecule, which remains active as long as the GTP-bound α subunit remains bound to it. Hence, the most obvious interpretation of the reduced bump amplitude in *Gaq* mutants is that only one or very few PLC molecules are activated per photon and that, normally, several PLC molecules must be activated in order to generate a fully amplified bump. If this is the case, one would predict that bump amplitude should also be reduced in *norpA* hypomorphs with sufficiently reduced

levels of PLC. Although independent laboratories have reported that bumps in *norpA* mutants are normal in amplitude (Cook et al., 2000; Scott and Zuker, 1998), we systematically investigated quantum bumps in a range of *norpA* alleles, including all those where quantum bumps had been measured previously.

As previously reported (Cook et al., 2000; Scott and Zuker, 1998), immediately on establishing the whole-cell configuration in photoreceptors from *norpA* mutants, there is an ongoing noisy inward current that slowly decays in the dark to reveal first isolated bump-like events and then a quiet baseline. This constitutive current was typically ∼30 pA in amplitude, took as long as 15 min to subside in the most severe alleles, and probably represents the summation of randomly occurring quantum bumps with extremely long latencies initiated by red light used for observation and/or by spontaneous G protein activation. After reaching baseline, bumps or macroscopic responses could be induced by calibrated light flashes, but bump latencies were greatly prolonged, resulting in responses which lasted for several minutes in the most severe alleles (Figure 4). In contrast to *Gaq¹*, however, Q.E. was generally similar to WT, except in the most severe alleles, such as *norpA^P12*. This behavior is believed to reflect the role of PLC as an obligatory GTP-ase accelerating protein (GAP) required for inactivation of the GTP-bound Gq α subunit (Cook et al., 2000; Scott and Zuker, 1998). Thus, Gq α subunits are activated normally in these mutants but remain active indefinitely until they finally encounter a rare PLC molecule, resulting in a greatly delayed cycle of activation and deactivation.

Thus far, these results and interpretation are in close agreement with previous studies; however, once again we found that quantum bumps in all eight *norpA* alleles tested were much smaller than those measured in WT but similar to those recorded in *Gaq¹* (Figure 4).

The "spontaneous" bumps recorded at the end of the decay period were quantitatively indistinguishable from those induced by calibrated light flashes after a quiet baseline had been reached. There were small but significant differences between different alleles, with mean bump amplitudes ranging from only ∼1.5 pA in *norpA^P12* (one of the most severe alleles) to ∼2.7 pA in *norpA^C1094S* and *norpA^P45*.

Quantum Bump Amplitude in *norpA* and *Gaq¹* Is ATP Dependent

Under our experimental conditions, there is a consistent several-fold reduction in bump amplitude in *Gaq¹*, *Gβe¹*, and eight independent alleles of *norpA*, strongly suggesting that WT quantum bumps require activation of several G protein and PLC molecules. Why then did previous authors report that bump amplitude and kinetics were normal in the same mutants? In the recordings of Zuker and colleagues, the intracellular solution in the electrode contained no nucleotide additives, such as ATP or GTP, which we routinely included in our recordings from these metabolically sensitive cells. In the absence of these additives, we previously found that, after several minutes of whole-cell recording, the light-sensitive channels open spontaneously, generating a so-called rundown current (RDC), following which sensi-
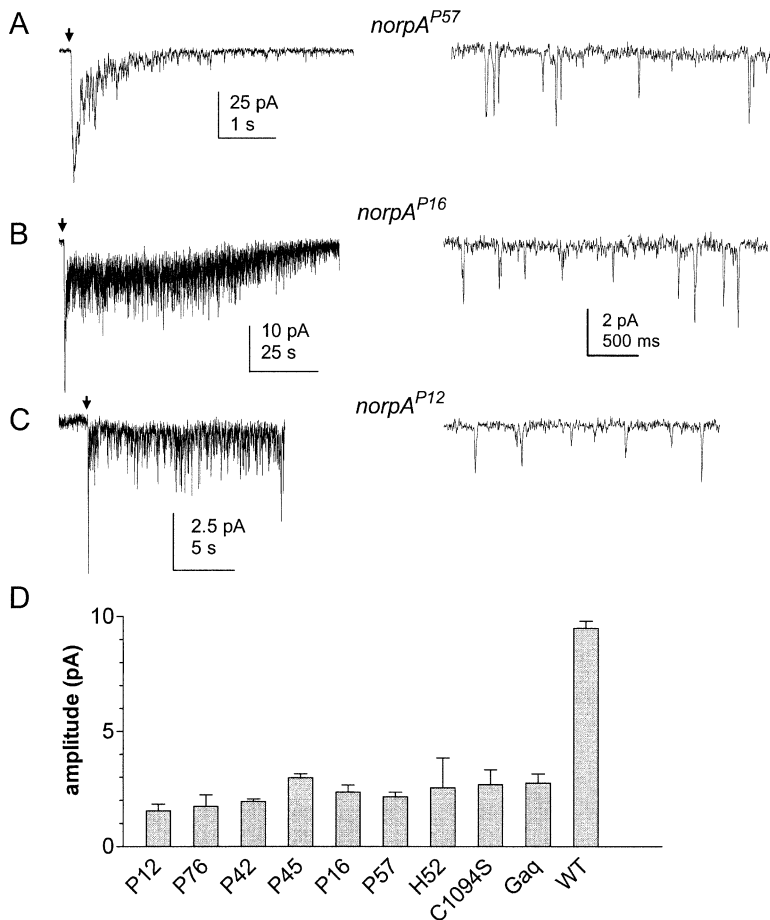
A

$norpA^{P57}$

25 pA
1 s

B

$norpA^{P16}$

10 pA
25 s

2 pA
500 ms

C

$norpA^{P12}$

2.5 pA
5 s

D

Figure 4. Quantum Bumps in norpA

(A–C) Response in three representative hypomorphic norpA alleles, including (A), the most sensitive tested ($norpA^{P57}$); (B), an intermediate hypomorph ($norpA^{P16}$); and (C), one of the most severe ($norpA^{P12}$). Left hand traces show macroscopic responses to brief flashes (arrows) containing $\sim 10^3$ WT effective photons ($norpA^{P57}$), 10^5 photons ($norpA^{P16}$), and 10^6 photons ($norpA^{P12}$). Right hand traces show trains of single bumps selected from the tails of responses to dimmer flashes containing ~ 20 effective photons ($norpA^{P57}$); 40 photons ($norpA^{P16}$), and 4000 photons ($norpA^{P12}$), respectively.

(D) Bar graph of bump amplitudes in eight norpA alleles, compared to Gaq^1 and WT. Mean $\pm$ SD, n = 3–11 cells.

tivity to light is severely attenuated (Hardie and Minke, 1994). Nevertheless, stable recordings can usually be obtained for several minutes in WT and often longer in $G\alpha q^1$ and norpA, before the onset of RDC. In recordings made under these conditions, we found that bump amplitudes in both $G\alpha q^1$ and norpA mutants increased to values approaching those found in WT (Figure 5). By contrast, in WT flies, omitting nucleotides from the electrode had virtually no effect on bump amplitude until the onset of RDC, so that under these conditions $G\alpha q^1$ and norpA bumps would indeed appear very similar to WT, as previously reported.

Our nucleotide additives included ATP (4 mM), GTP (0.4 mM), and NAD (1 mM). To determine which was critical for recording small bumps, we tested each in turn. Recordings in $G\alpha q^1$, using GTP or NAD, on their own or in combination, yielded bumps as large as those recorded with no additives, but recordings made with 4 mM ATP invariably resulted in small bumps. Depending on endogenous ATP levels, either adding ATP could suppress bump amplitude, or omitting ATP could result in an artifactual increase. To determine which was more likely, we first estimated the dose dependence by varying the ATP concentration in the electrode. In both Gaq^1 and $norpA^{P16}$, bumps only became significantly larger when ATP was lowered below 1 mM (Figure 5D). Since resting ATP concentrations in most cells are

greater than 1 mM, with the only measurements of ATP in an insect photoreceptor providing a value of ~ 3 mM in drone bee (Brazitikos and Tsacopoulos, 1991), it seems likely that small bumps reflect the physiological situation. As an additional test, we also started recording quantum bumps within seconds of establishing the whole-cell configuration. In every case, when the electrode solution contained no ATP (n = 5), quantum bumps recorded during the first 1–2 min in $G\alpha q^1$ were of a similar small size to those recorded under our control solutions but then increased to values approaching those seen in WT (Figure 5C). By contrast, in recordings made with ATP, small bumps were always apparent from the beginning.

Diacylglycerol Kinase Regulates Bump Amplitude in Gaq^1 and norpA

The increase in bump size seen on removing ATP in $G\alpha q^1$ and norpA suggested that bump amplitude might be regulated by an ATP-dependent kinase. One such candidate is diacylglycerol kinase (DGK), encoded by the retinal degeneration A (rdgA) gene and which has recently been implicated in response termination (Raghu et al., 2000b). To test whether failure of DGK, which metabolizes DAG to form phosphatidic acid (PA), might be responsible for the enhanced bumps seen in the
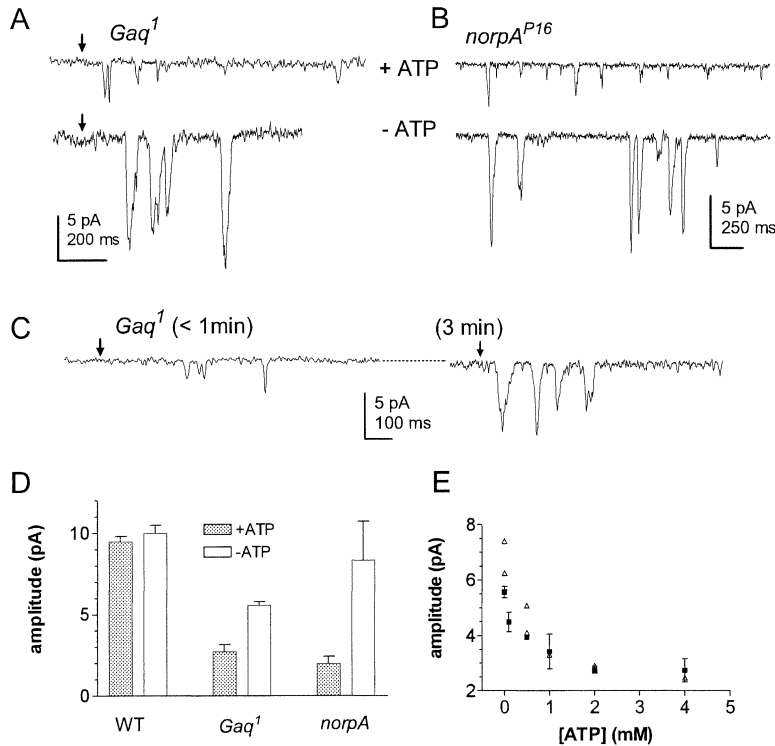
Figure 5. ATP Dependence of Quantum Bumps

(A and B) Quantum bumps induced by flashes (arrows) in $G\alpha q^1$ (A) and spontaneous bumps in $norpA^{P16}$ (B) with and without (+/− ATP) nucleotide additives. A similarly dramatic increase in bump amplitude was also seen in $norpA^{P57}$ and $norpA^{P12}$ (other alleles not tested). (C) Quantum bumps from another $G\alpha q^1$ cell recorded without nucleotides. Left: during the first minute after establishing whole-cell; right: ∼3 min later. (D) Bump amplitudes with and without nucleotide additives: mean ± SD, n ≥ 4 cells (for *norpA*, results from P57, P12, and P16 have been pooled); (E) bump amplitude in Gaq^1 (■ mean ± SD, n ≥ 3 cells for each concentration); and $norpA^{P16}$ (△ individual cells) as a function of concentration of ATP in the electrode.

absence of ATP, we generated double mutants of both $G\alpha q^1$ and *norpA* with *rdgA*.

rdgA;Gaq¹

The most severe DGK allele, $rdgA^1$, undergoes massive, early onset retinal degeneration (Masai et al., 1993) associated with constitutive activation of the light-sensitive TRP channels and a virtual lack of light response (Raghu et al., 2000b). We also failed to detect any response in $rdgA^1;G\alpha q^1$ double mutants, therefore, we tested the weaker allele, $rdgA^3$. As in $rdgA^1$, we found that the light-sensitive channels in $rdgA^3$ were constitutively active in the dark and, although robust light responses could be recorded, quantum bumps could not be detected (data not shown). By contrast, in $rdgA^3;G\alpha q^1$, there was usually no longer significant constitutive activity, allowing bumps to be clearly resolved. Consistent with identification of DGK as the critical ATP-dependent factor, the bumps were restored to near WT levels in amplitude and also showed a variable defect in termination (Figure 6). Significantly, Q.E. in $rdgA^3;Gaq^1$ was also enhanced at least 10-fold (17 ± 12-fold, n = 7) with respect to Gaq^1, again implying that most activated G proteins in the single Gaq^1 mutant fail to generate a bump at all. Since G protein α subunits are believed to remain active until they encounter PLC, this in turn implies that, with normal DGK activity, activation of a single PLC molecule may usually be insufficient to overcome the threshold for bump generation.

norpA,rdgA Double Mutants

As will be described in detail elsewhere, the *rdgA* retinal degeneration phenotype was largely rescued in *norpA,rdgA* double mutants, allowing analysis using the most severe *rdgA* allele. We generated double mutants using both $norpA^{P16}$, which has normal Q.E., and $norpA^{P12}$, which has a reduced Q.E, generating maximum responses of only ∼5 pA. As in the respective *norpA* single mutants, in both these *norpA,rdgA* double mutants there was a constitutive noisy inward current on establishing the whole-cell configuration, which then subsided in typical fashion over 10–15 min to leave single bumps which were greatly enhanced in amplitude and showed conspicuous and rather variable deactivation defects (Figure 7A). Bump amplitudes were now similar to WT (8–10 pA), and because of the deactivation defect, the current integrals were even larger (∼500 pA.ms)—more than ten times that seen in the respective *norpA* single mutants.

Macroscopic sensitivity was tested using flashes containing either ∼4000 or ∼10^6 effective photons. As in the single mutant controls, these flashes induced responses that lasted several minutes, but response amplitudes were greatly increased. In $norpA^{P16},rdgA^1$, a 10- to 20-fold increase in macroscopic responses appeared to be accounted for by the increase in bump integral currents; however, in $norpA^{P12},rdgA^1$, there also appeared to be an increase in Q.E, which together with the increase in bump integral resulted in a massive ∼100-fold potentiation of the response (Figure 7E). Overall, quantum bumps and macroscopic responses recorded in *norpA,rdgA* double mutants were similar to those seen in long-term recordings from *norpA* single mutants without ATP in the pipette (e.g., Figure 7B), supporting the identification of DGK as the ATP-dependent factor responsible for controlling bump amplitude in *norpA* and Gaq^1 mutants.
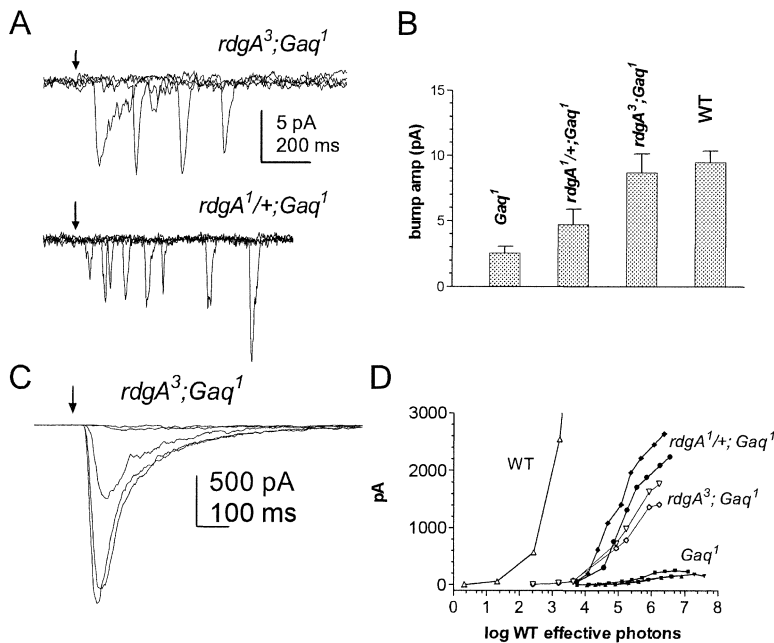
Figure 6. DAG Kinase Mutations Enhance Sensitivity in $G\alpha q^1$

(A) Quantum bumps recorded in response to brief flashes in $rdgA^3;G\alpha q^1$ and $rdgA^1/+;Gaq^1$.

(B) Bar graph summarizing mean bump amplitude in $rdgA^3;Gaq^1$ (n = 9) and $rdgA^1/+;Gaq^1$ (n = 10) compared to Gaq^1 and WT controls.

(C) Macroscopic responses to flashes of increasing intensity in $rdgA^3;Gaq^1$ (range 500 to 2×10^6 WT effective photons).

(D) Peak responses plotted against number of WT effective photons for $rdgA^1/+;Gaq^1$ and $rdgA^3;Gaq^1$ photoreceptors (two cells each) compared to Gaq^1 and WT.

This was further supported by finding that omitting ATP from the pipette no longer had detectable effects on bump amplitude or sensitivity in *norpA,rdgA* double mutants (Figure 7).

Reduction in PIP_2 Does Not Account for the Effect of the *rdgA* Mutation in *norpA* and Gaq^1

The dramatic enhancement of bump amplitude and Q.E. in *norpA* and $G\alpha q^1$ by mutations in DGK is potentially strong evidence for an excitatory role of DAG in phototransduction. However, conversion of DAG to PA by DGK is also the first step in regeneration of PIP_2, so that PIP_2 levels may also be reduced in *rdgA* mutants. Conceivably, this could result in an increase in bump amplitude if excitation were mediated by a reduction in PIP_2. One argument against this possibility is that bump amplitude was rapidly increased by simply omitting ATP from the electrode, although in vivo measurements have shown that there was no detectable reduction of microvillar PIP_2 in *norpA* flies on this timescale (R.C.H., unpublished data). In addition, we used two further approaches to exclude this possibility.

First, we reasoned that it would be very unlikely that reducing DGK gene dosage would lead to a reduction in equilibrium dark-adapted PIP_2 levels, particularly on *norpA* or *Gaq* backgrounds where the main pathway for PIP_2 hydrolysis is severely compromised. In support of this, by themselves, $rdgA^1/+$ heterozygotes had no discernible phenotype in bump amplitude, sensitivity, or response kinetics (data not shown). However, if rapid metabolism of DAG is critically involved in determining threshold and bump amplitude in *norpA* and $G\alpha q^1$, then the modest reduction in DGK achieved by halving gene dosage might be expected to enhance bump amplitude. In confirmation of this, photoreceptors from both *norpA,rdgA¹/+* and $rdgA^1/+;G\alpha q^1$ flies showed a robust and significant ~2-fold increase in bump amplitude (Fig-

ures 6 and 7) and, in the case of $rdgA^1/+;G\alpha q^1$, an ~20-fold increase in Q.E. compared to $G\alpha q^1$ controls.

Second, we explored the effect of a mutation in the enzyme catalyzing the next step of PIP_2 resynthesis, namely, CDP-diacylglycerol synthase (CDS). PIP_2 levels in *cds* mutants should be more severely compromised than in *rdgA*, not only because *cds* is a protein null mutant (Wu et al., 1995) while $rdgA^3$ in particular is a relatively weak hypomorph, but also because PA can be synthesized de novo, while CDS is an obligatory enzyme for PIP_2 synthesis. Indeed, in *cds* mutants, sensitivity to light can be irreversibly reduced by conditioning light flashes, indicating an irreversible loss of microvillar PIP_2, while Q.E. is often reduced and bump latency prolonged even in dark-adapted flies, suggestive of reduced steady-state PIP_2 levels (Hardie et al., 2001; Wu et al., 1995). We first confirmed that PIP_2 was irreversibly depleted in *cds* mutants by monitoring PIP_2 levels with a genetically targetted PIP_2-sensitive ion channel, Kir2.1 (Figure 8) as previously reported (Hardie et al., 2001). We then generated $Gaq^1;cds$ double mutants to test whether *cds* could mimic the effect of the *rdgA* mutation. However, in marked contrast to $rdgA^3;Gaq^1$, quantum bumps in $Gaq^1;cds$ were at least as small (1.9 ± 0.2 pA, n = 3) as in Gaq^1 controls (Figure 8). Furthermore, Q.E. was yet further reduced (at least 10-fold), suggesting that the reduced PIP_2 levels may have prevented many activated G proteins from generating sufficient DAG to reach threshold for bump generation. In addition, we also recorded quantum bumps in *cds* mutants on an otherwise WT background before and after conditioning illumination calibrated to partially deplete PIP_2. In all cases, quantum bump amplitudes were decreased in amplitude (Figure 8).

We conclude that the enhancement of sensitivity by the *rdgA* mutation cannot be attributed to PIP_2 depletion but is fully consistent with an effect on dynamic DAG levels.
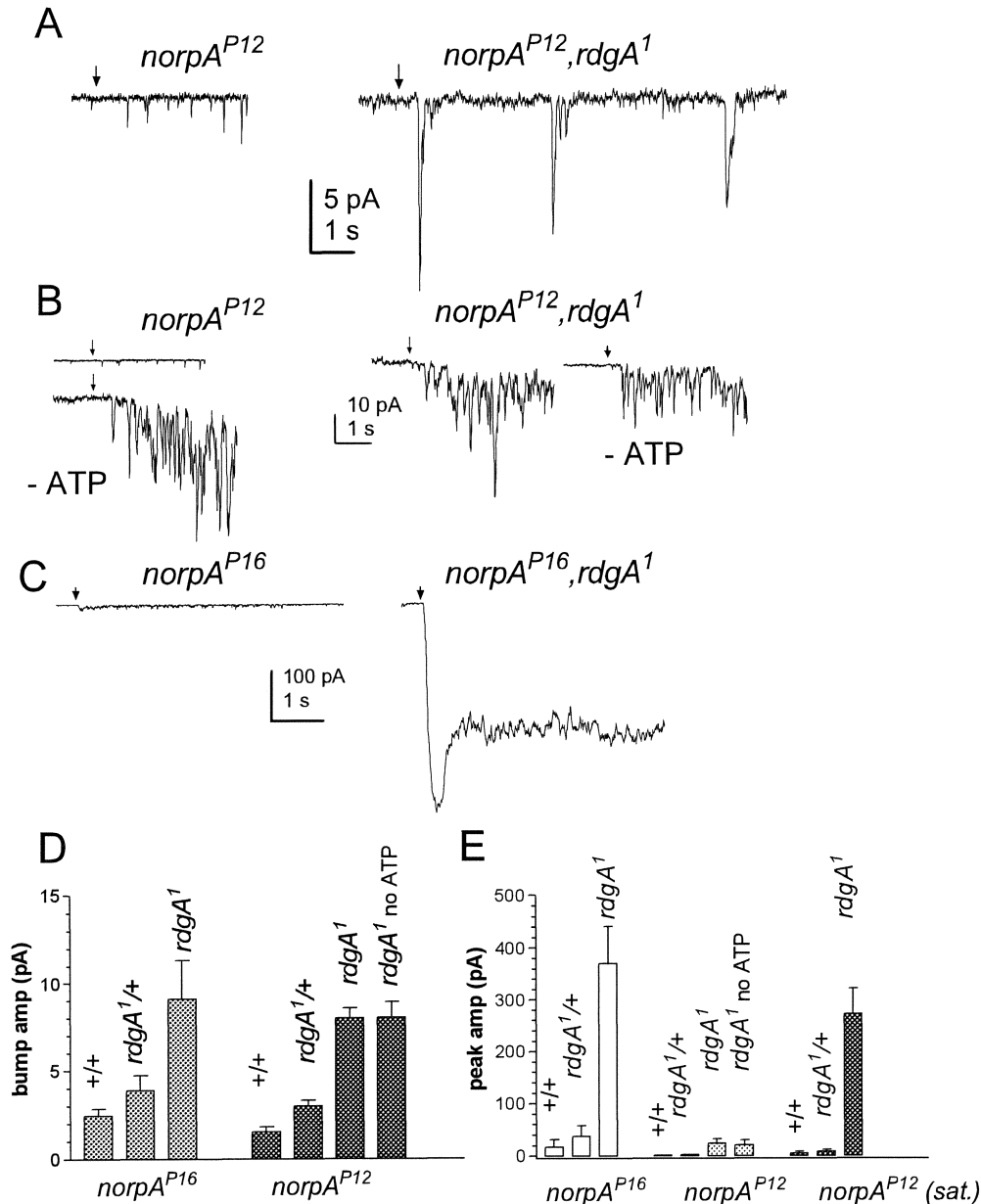
Figure 7. Massive Facilitation in *norpA* by DAG Kinase Mutation

(A) Quantum bumps elicited by flash containing ∼4000 effective photons in *norpA*^{P12} (left) and 120 photons in *norpA*^{P12},*rdgA* (right). Bumps are greatly increased in size on the *rdgA* background, often terminating with irregular, noisy tails.

(B and C) Macroscopic responses elicited by flashes containing ∼4000 WT effective photons: responses in both *norpA*^{P12} ([B], left) and *norpA*^{P16} ([C], left) are greatly facilitated on an *rdgA* background (right). Note different scales for *P12* and *P16*. Responses in *norpA*^{P12} and *norpA*^{P12},*rdgA* are also shown with electrodes containing no ATP (−ATP).

(D) Bar graph summarizing effect of homozygous and heterozygous *rdgA*^{1} background on bump amplitude (n = 5 cells ± SD, >50 bumps in each cell; +/+ = single mutant controls).

(E) Bar graph summarizing effect of *rdgA* backgrounds on macroscopic peak responses to flashes containing ∼4000 photons and also (*norpA*^{P12} only) a saturating flash containing ∼2 × 10^{6} effective photons. n = 5 cells.

Discussion

The current view that amplification in the *Drosophila* phototransduction cascade is mediated downstream of PLC (Scott and Zuker, 1998) is based largely on reports that bump amplitude and timecourse were unaffected in mutants of G protein or PLC (Pak et al., 1976; Scott

et al., 1995; Scott and Zuker, 1998; reviewed by Hardie and Raghu, 2001). However, after testing an extensive range of mutants of both G protein and PLC, we found that in all cases quantum bump amplitude was greatly reduced. Although these results seemed to directly contradict earlier studies, we found that when ATP was omitted from the electrode, bump amplitude ap-
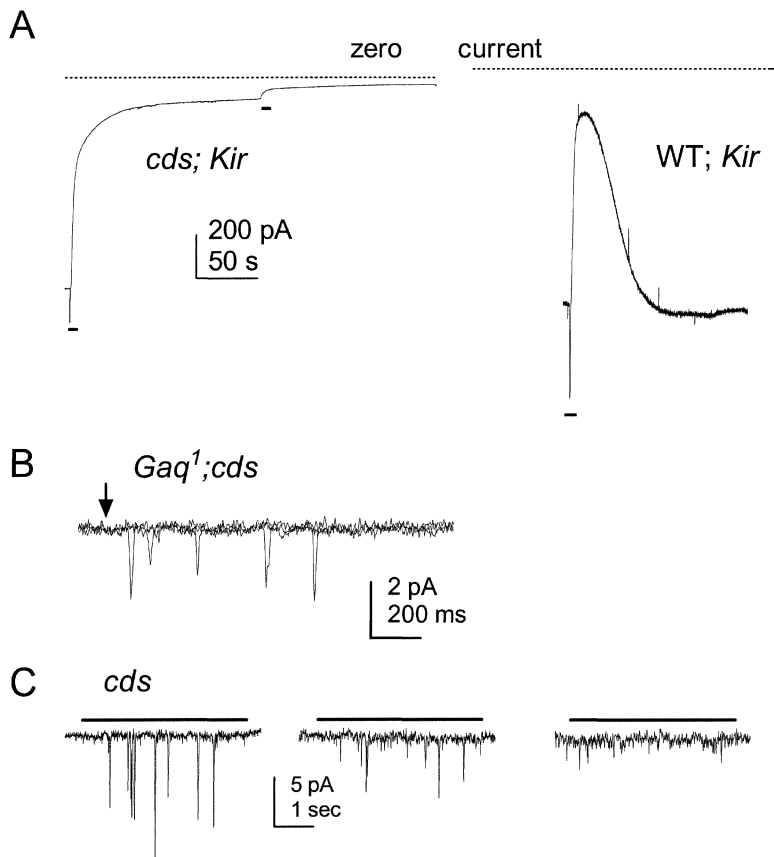
A

zero   current

cds; Kir

WT; Kir

200 pA
50 s

B   *Gaq¹;cds*

2 pA
200 ms

C   *cds*

5 pA
1 sec

Figure 8. The *cds* Mutation Does Not Enhance Quantum Bump Amplitude

(A) Irreversible depletion of PIP$_2$ in *cds* mutants. In flies expressing the PIP$_2$-sensitive ion inward rectifier channel Kir2.1, a large constitutive inward current (dotted lines indicate zero current levels) represents the activity of PIP$_2$ bound Kir2.1 channels in the microvillar membrane. In control (WT;*Kir*) flies, the Kir current is suppressed by a 5 s light step (bar) within 1–2 s, representing loss of microvillar PIP$_2$, but recovers over ∼1 min as PIP$_2$ is resynthesized. In *cds;Kir* flies, however, the loss of PIP$_2$ is irreversible (recordings made in the presence of 20 μM La^{3+} to block TRP channels).

(B) Quantum bumps induced by flashes (arrow, 4000 WT effective photons, four superimposed traces) in a *Gaq¹;cds* double mutant have the small size typical of *Gaq¹* (cf. Figure 3).

(C) Quantum bumps induced by dim light steps (bars) in *cds* mutant (otherwise WT background) before (left) and after successive incremental partial depletion of PIP$_2$ by conditioning light flashes (middle and right).

proached WT values. This almost certainly accounts for the apparently conflicting results of Scott et al. (1995) and Scott and Zuker (1998). Cook et al. (2000) analyzed bump in detail only from *norpA^{C1094S}* flies, which we found to have among the largest bumps of the *norpA* alleles tested (∼3 pA with occasional bumps as large as 10 pA). Since these authors excluded events less than 3 pA in amplitude from their analysis, their results are not necessarily in conflict with ours, and this lab now confirms that small bumps are found, e.g., with *norpA^{P57}* under similar recording conditions (B. Minke, personal communication). Most significantly, we were able to closely mimic the effect of removing ATP by mutations in the *rdgA* gene encoding DGK. As argued in more detail below, these results indicate that amplification in *Drosophila* is critically dependent on activation of multiple G protein and PLC molecules and identify DGK as a key enzyme regulating bump amplitude and inactivation.

A number of independent arguments exclude the possibility that the reduced bump amplitudes seen in Gq and PLC hypomorphs were due to defective protein rather than low protein levels. First, bump amplitude was reduced irrespective of whether G protein or PLC was mutated. Secondly, the *Gaq¹* mutation was effectively mimicked both by RNAi and also a mutation in the Gβ subunit. Third, four of the *norpA* alleles were missense mutations resulting in a premature stop codon (Table 1). Since the resulting truncated protein would probably be completely inactive in these cases, the residual functional protein is likely to derive from read-through of the stop codon and hence constitute a WT

protein differing by at most one amino acid (Pearn et al., 1996). Finally, we observed spontaneous dark events in WT flies that were indistinguishable from *norpA* and *Gaq¹* bumps, which seem likely to represent spontaneous activation of Gq molecules (and consequently PLC) in the absence of illumination.

The question also arises whether the electrode solu-

Table 1. Mutants Used

Allele	Description of Mutation
Gaq¹	splice acceptor site leading to in-frame deletion of residues 154–156. ∼1% protein[1]
Gβe¹	Tyr293 to Cys <0.5% protein[2]
GaqRNAi	double stranded RNA under UAS control[3]
norpA^{P12}	premature stop codon (UAG 240)[4]
norpA^{P42}	premature stop codon (UAG 622)[4]
norpA^{P45}	premature stop codon (UAG 748)[4]
norpA^{P76}	premature stop codon (UGA 606)[4]
norpA^{P57}	Gly768 to Asp[4]
norpA^{C1094S}	*norpA* transgene with targeted mutation in PDZ binding motif expressed on *norpA^{P24}* background[5]
norpA^{H52}	temperature-sensitive, near null at 37°C[6]
norpA^{P16}	Arg362 to Cys[6]
rdgA¹	a.k.a. *rdgA^{BS12}*, most severe allele <5% DGK activity; degenerates on day of eclosion[7]
rdgA³	a.k.a *rdgA^{PC47}*, less severe allele; degenerates within 1 week[8]
cds¹	P insert at 66B, protein null[9]

References: 1, Scott et al. (1995); 2, Dolph et al. (1994); 3, Kalidas and Smith, (2002); 4, Pearn et al. (1996); 5, Shieh et al. (1997); 6, Deland and Pak (1973); 7, Masai et al. (1993); 8, Harris and Stark (1977); 9, Wu et al. (1995).

tion, containing 4 mM ATP, might artifactually have suppressed bump amplitudes. Because ATP had to be reduced below 1 mM in order to increase bump amplitude and since bumps were always small immediately after establishing the whole-cell configuration, irrespective of the ATP concentration, we believe that our measurements closely reflect the physiological situation. This accords with the only available evidence from in situ recordings. Thus, from noise analysis of intracellular recordings in the larger fly, *Musca*, Minke and Stephenson (1985) concluded that the underlying events responsible for voltage noise induced by fluoride were about four times smaller than those induced by light. Since fluoride was assumed to directly activate G protein, these authors already concluded that there was likely to be amplification at the level of G protein.

Although Minke and Stephenson's findings were subsequently overlooked in view of the more direct measurements of voltage-clamped quantum bumps in *norpA* and *Gaq*, we now conclude that amplification in *Drosophila* phototransduction is indeed critically dependent upon activation of multiple G proteins and PLC molecules. Assuming linear summation, the difference in bump current integral between WT and the most severe *Gq* and *norpA* hypomorphs suggests that at least five PLCs need be activated in order to generate a typical WT bump. Since quantum bumps in *Drosophila* correspond to the simultaneous opening of only about 15 channels at the peak of the bump, amplification at the level of the G protein may in fact represent the major component of amplification in *Drosophila*. Interestingly, a similar number of G proteins (about eight) are believed to be activated in *Limulus* ventral photoreceptors (Kirkwood and Lisman, 1994), although amplification downstream of PLC dominates in these cells—probably by very distinct mechanisms.

Significantly, lowering ATP did not further increase bump amplitude in WT photoreceptors, suggesting that the bump-generating machinery is saturated in dark-adapted photoreceptors. This can be understood if quantum bumps represent activation of all available channels within the microvillus. The suggestion that the unit of signaling underlying the quantum bump is the microvillus (Hochstrate and Hamdorf, 1990; Howard et al., 1987) is also consistent with the finding that the number of channels activated during the quantum bump corresponds closely to the number predicted per microvillus from quantitative Western analysis (Huber et al., 1996).

Is DAG the Excitatory Messenger?

The essential role of PLC in *Drosophila* phototransduction is well established (Bloomquist et al., 1988), but the downstream mechanisms responsible for gating the light-sensitive channels remain controversial. Accumulating evidence, including the lack of phenotype in mutants of the only $InsP_3$ receptor gene known in *Drosophila* (Acharya et al., 1997; Raghu et al., 2000a), suggests that $InsP_3$ may not be involved in excitation (review by Hardie and Raghu, 2001). Additional consequences of PLC activation include generation of DAG and a reduction in PIP_2, both of which are also currently under discussion as excitatory messengers for some vertebrate TRP homologs (review Clapham et al., 2001). Both TRP and TRPL channels can be activated by polyunsaturated

fatty acids (PUFAs), which might be released from DAG by a DAG lipase (Chyb et al., 1999). More recently, Estacion et al. (2001) found that heterologously expressed TRPL channels could also be activated by DAG, raising the possibility that DAG may be the endogenous transmitter, with PUFAs mimicking their action. However, Estacion et al. (2001) suggested that at least some of the actions of DAG and PUFAs may be indirect via activation of endogenous PLC and reported that activity of TRPL channels in patches was suppressed by application of PIP_2, suggesting PIP_2 depletion as a potential contributory factor to channel gating.

Our analysis of the DGK mutant *rdgA* now provides strong independent evidence for an excitatory role for DAG. We previously reported that TRP and TRPL channels in *rdgA* mutants are constitutively active and that degeneration was largely prevented in *rdgA;trp* double mutants (Raghu et al., 2000b). The response to light was also rescued in *rdgA;trp*, revealing a deactivation defect, suggesting a role for DGK in response termination, at least with respect to TRPL channels. These results would be consistent with a role for DAG in excitation; however, DGK is also the first enzyme in the PIP_2 recycling pathway, so that PIP_2 levels may also be affected in the *rdgA* mutant. Furthermore, the remaining TRPL channels were still constitutively active in the *rdgA;trp* double mutant, and light responses could only be compared to controls in pupae during a very narrow developmental time window.

In the present study, we generated *rdgA* double mutants with both $G\alpha q^1$ and *norpA*, allowing analysis in adult flies with intact TRP and TRPL channel function. Strikingly, bump amplitudes in both *norpA* and $G\alpha q^1$ were restored to WT levels by *rdgA* mutations, also showing defects in inactivation. In addition, Q.E. was enhanced, resulting in some cases in massive (~100-fold) overall increases in sensitivity. Since our evidence indicates that these *rdgA* phenotypes are not due to reduced PIP_2 levels, we interpret this as compelling evidence for the role of DAG as messenger of excitation. DAG levels are dynamically determined by the balance between PLC activity (generating DAG from PIP_2) and DGK activity (converting DAG to PA). It seems that when only one PLC molecule is activated, DAG is metabolized too quickly for threshold levels to be reached, except perhaps in the immediate vicinity of the activated PLC. However, if DGK is inactivated by the *rdgA* mutation or by depleting ATP, then DAG can reach threshold more readily and also diffuse to activate more distant channels so that Q.E. and quantum bump amplitudes approach WT values. (Note that the reduction in Q.E. in $G\alpha q^1$ was previously attributed to most activated rhodopsins being inactivated before they had a chance to encounter a rare G protein [Scott and Zuker, 1998]; our results suggest that a major factor in the reduction of Q.E. is that, despite activating a PLC, most single activated G proteins result in insufficient DAG generation to overcome bump threshold). Despite these arguments, we would not exclude a contributory role of PIP_2 to channel regulation. For example, it is conceivable that channels are bound to PIP_2 in the closed state, that channel activation involves exchange of PIP_2 for DAG, and that channel closure following excitation may involve rebinding of PIP_2.

A Conceptual Model for Bump Generation

Multiple, sequential G protein activation is well established as a mechanism of amplification in vertebrate phototransduction. Although we have concluded that multiple G protein activation is also required for amplification in *Drosophila*, the molecular strategies remain qualitatively and quantitatively distinct (reviewed by Hardie and Raghu, 2001). In rods, each activated G protein rapidly encounters a PDE, which immediately begins to hydrolyze cGMP. The cGMP concentration is sensed continuously by the light-sensitive channels, which progressively close as upward of 100 G protein and PDE molecules are recruited by random diffusional encounters with rhodopsin. This gives rise to quantum bumps with very short latencies, but which rise gradually over a time course of ~1 s in toad or ~100 ms in mammalian rods. In *Drosophila* and other microvillar photoreceptors, there is a finite and variable latency (~20–100 ms in *Drosophila*) followed by an abrupt (~10 ms) rising phase, indicative of a threshold and positive feedback—features not found in vertebrate photoreceptors. At rest in the dark, the channels are closed, and the latency presumably represents the time taken for diffusional encounters of, perhaps, five to ten G proteins with rhodopsin and PLC and then for sufficient second messenger (DAG or its PUFA metabolites) to accumulate to activate the first channel. Because of the restricted volume of the microvillus, Ca^{2+} influx via the first channel raises Ca^{2+} rapidly throughout the microvillus. If other channels in the microvillus are already exposed to subthreshold concentrations of DAG generated by other PLC molecules, we propose that the raised Ca^{2+} sensitizes these channels (e.g., by increasing the affinity of the channel for DAG/PUFA), resulting in an explosive positive feedback which activates all or most of the channels in the microvillus over a time course of ~10 ms. Ca^{2+}, which reaches concentrations of at least 200 μM (Oberwinkler and Stavenga, 2000), then mediates negative feedback terminating the bump. Finally, there is believed to be a refractory period lasting ~100 ms, while the Ca^{2+} is cleared by diffusion and/or Na^+/Ca^{2+} exchange and necessary biochemical steps of inactivation (e.g., Rh-arrestin binding, GTPase activity, clearance of DAG, resynthesis of PIP_2) run their course (reviewed by Hardie and Raghu, 2001).

Concluding Remarks

In summary, our results indicate that amplification in *Drosophila* phototransduction is critically dependent upon activation of multiple G proteins and PLC molecules, identify DGK as a key enzyme regulating amplification, and strongly support the identification of DAG as messenger of excitation in *Drosophila* phototransduction. PLC has long been recognized as the effector enzyme in invertebrate phototransduction, playing an analogous role to PDE in vertebrate rods (although PLC generates the active transmitter, while PDE degrades it). Our results now suggest that DGK is the key enzyme controlling the supply of second messenger in *Drosophila* and thus plays an analogous role to guanylate cyclase. Ca^{2+}-dependent regulation of guanylate cyclase is a key mechanism of light adaptation and response termination in vertebrate rods (reviewed by Pugh et al.,

1999). It will be interesting to see whether similar regulation of DGK is involved in regulating sensitivity and kinetics during light adaptation in *Drosophila*.

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