Rescue of Light Responses in the Drosophila “Null” Phospholipase C Mutant, norpA<sup>P24</sup>, by the Diacylglycerol Kinase Mutant, rdgA, and by Metabolic Inhibition<sup>k</sup>

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Roger C. Hardie<sup>‡</sup>, Fernando Martin, Sylvester Chyb<sup>§</sup>, and Padinjat Raghut<sup>†</sup>

From the Cambridge University Department of Anatomy, Cambridge CB2 3DY, United Kingdom

Light responses in Drosophila are reportedly abolished in severe mutants of the phospholipase C (PLC) gene, norpA. However, on establishing the whole-cell recording configuration in photoreceptors of the supposed null allele, norpA<sup>P24</sup>, we detected a small (<15 pA) inward current that represented spontaneous light channel activity. The current decayed during ~20 min, after which tiny residual responses (<2 pA) were elicited by intense flashes. Both spontaneous currents and light responses appeared to be mediated by residual PLC activity, because they were enhanced by impairing diacylglycerol (DAG) kinase function by mutation (rdgA) or by restricting ATP but were reduced or abolished by a mutation of the PLC-specific G<sub>α</sub> subunit. It was reported recently that metabolic inhibition activated the light-sensitive transient receptor potential and transient receptor potential-like channels, even in norpA<sup>P24</sup>, leading to the conclusion that this action was independent of PLC (Agam, K., von Campenhausen, M., Levy, S., Ben-Ami, H. C., Cook, B., Kirschfeld, K., and Minke, B. (2000) J. Neurosci. 20, 5748–5755). However, we found that channel activation by metabolic inhibitors in norpA<sup>P24</sup> was strictly dependent on the residual PLC activity underlying the spontaneous current, because the inhibitors failed to activate any channels after the spontaneous current had decayed. By contrast, polyunsaturated fatty acids invariably activated the channels independently of PLC. The results strongly support the obligatory requirement for PLC and DAG in Drosophila phototransduction, suggesting that activation by metabolic inhibition is primarily because of the failure of diacylglycerol kinase, and are consistent with the proposal that polyunsaturated fatty acids, which are potential DAG metabolites, act directly on the channels.

Phototransduction in the microvillar photoreceptors of invertebrates is mediated by the inositol lipid or phosphoinositide signaling cascade. Large because of its molecular genetic potential, Drosophila has become an important model system not only for phototransduction but for inositol lipid signaling in general (1–3). The generally accepted mechanism of excitation conforms to the canonical phosphoinositide cascade (see Fig. 8). After photoisomerization in the microvillar membrane, rhodopsin activates a G<sub>α</sub> protein which, in turn, binds to and activates phospholipase C (PLC<sup>41</sup> isoform), resulting in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) to generate inositol 1,4,5-trisphosphate and DAG. By a still unknown mechanism, this process activates at least two classes of light-sensitive channels in the microvilli, encoded by the transient receptor potential potential (trp) and trp-like (trpL) genes, which are the prototypical members of the large TRP ion channel superfamily (4, 5). Recent evidence suggests that lipid products of PIP<sub>2</sub> hydrolysis are the messengers of excitation (6, 7), but this is still a subject of controversy (3, 8–10).

The key argument for the conclusion that phototransduction in Drosophila is an obligatory PLC-signaling cascade is the finding that the norpA (no receptor potential A) gene encodes an eye-enriched PLC<sub>β</sub> and that severe mutations in norpA abolished the response to light (11–13). Less severe hypomorphic norpA mutants show reduced sensitivity with dramatically slowed kinetics (14–16) and reduced amplification, as indicated by the small amplitude of responses to single photons known as quantum bumps (17). We recently discovered that sensitivity in norpA hypomorphs could be greatly facilitated by mutations in the retinal degeneration A (rdgA) gene and also by depletion of cytosolic ATP (17). This supports the hypothesis that DAG or its metabolites are excitatory messengers, because the rdgA gene encodes diacylglycerol kinase (DGK) (18), which is the major enzyme controlling DAG in most cells (19). In the present study we show that even the supposedly null mutant norpA<sup>P24</sup> has a residual sensitivity to light, which can be massively enhanced by either ATP depletion or the rdgA mutation but still appears to be mediated by PLC. In light of these findings we repeated some key experiments, which assumed that norpA<sup>P24</sup> was functionally null. In particular we found that contrary to a recent report (8), activation of the light-sensitive channels by metabolic inhibitors is strictly dependent on PLC activity, and we present evidence here that this represents the failure of DGK. By contrast, excitation of the channels by PUFAs (6), suggested previously to be caused indirectly by metabolic inhibition (8) or by the activation of endogenous PLC (20), appeared to be independent of PLC activity and seems likely to represent a direct effect on the channels or their lipid environment.

<sup>‡</sup>To whom correspondence should be addressed: Cambridge University, Dept. of Anatomy, Downing St., Cambridge CB2 3DY, United Kingdom. Tel.: 44-1223-339771; Fax: 44-1223-333786; E-mail: rch14@hermes.cam.ac.uk.

<sup>§</sup>Present address: Imperial College, University of London, Wye Campus, Wye, Kent TN25 5AH, United Kingdom.

<sup>†</sup>IBBSC David Phillips Research Fellow. Present address: Babraham Institute, Babraham, Cambridge CB2 4AT, United Kingdom.

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<sup>1</sup>The abbreviations used are: PLC, phospholipase C; DAG, diacylglycerol; PUFAs, polyunsaturated fatty acids; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; DGK, diacylglycerol kinase; WT, wild type; LNA, linolenic acid; CCCP, carbonyl cyanide m-chlorophenylhydrazone; DNP, 2,4-dinitrophenol; QE, quantum efficiency; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid; TRP, transient receptor potential; TRPL, TRP-like; PI, phosphatidylinositol.
**EXPERIMENTAL PROCEDURES**

Flies—Flies (Drosophila melanogaster) were raised on standard medium in the dark at 25°C. The wild type (WT) strain was white-eyed Oregon R. Mutant alleles included: Gαq3, a severe hypomorphic mutation (approximately 1% protein) of the eye-enriched G protein α subunit (21); norpAP24, a reportedly protein-null mutant of PLC with a 28-bp deletion (14); Dr1HC244/FM7C, a deficiency covering 3E8-4F11, which includes the entire norpA genomic region; rdgA1 (also termed rdgA282b), the most severe reported allele of an eye-specific DAG kinase with <5% DGK activity and complete degeneration on the day of eclosion (18); and sl2 and sl3, severe mutants of PLCγ (22).

Whole-cell Recordings—Dissociated photoreceptor clusters (ommatidia) were prepared as described previously (23, 24) from recently eclosed adult flies and transferred to a recording chamber on an inverted Nikon Diaphot microscope. The bath solution contained (in mM): 120 NaCl, 5 KCl, 10 TES, 1.5 CaCl2, 25 proline, and 5 alanine. Unless otherwise stated, intracellular solution was (in mM): 140 potassium gluconate, 10 TES, 2 MgCl2, 1 NAD, and 0.4 NaGTP. The pH of all solutions was adjusted to 7.15. To determine current-voltage relationships (Fig. 7), potassium gluconate was replaced with 125 mM cesium gluconate and 15 mM tetraethylammonium chloride to block K+ channels. Carbonyl cyanide m-chlorophenylhydrazone (CCCP), 2,4-dinitrophenol (DNP), and Ionolectin acid (LNA) were obtained from Sigma and were applied from a puffer pipette (tip diameter, 2–4 μm) positioned ~10 μm from the recorded cell. Whole-cell voltage clamp recordings were made at a holding potential of ~70 mV unless otherwise stated, using electrodes of approximately 10–15 MΩ resistance. Series resistance values were generally below 30 MΩ and were compensated to approximately 80% when recording macroscopic responses but not for collecting bumps. Data were sampled at 0.5–2 kHz and filtered at 100 Hz using Axopatch 1-D or 200B amplifiers and pCLAMP 6 or 8 software (Axon Instruments, Foster City, CA). Cells were stimulated via a green light-emitting diode. All intensities are expressed with respect to effectively absorbed photons in WT flies, which were estimated by counting quantum bumps at the lowest intensities and then calibrating relative intensities by using a linear photodiode. Quantum bumps were detected and analyzed using Mini-analysis (Synaptosoft, Decatur, GA). The quantum efficiency (QE) (equal to the percentage of absorbed photons eliciting a quantum bump) was estimated relative to WT by counting the number of quantum bumps in response to calibrated flashes (25), or when they could not always be clearly separated, by integrating the current under the entire response and dividing by the average bump integral current recorded in the same cell. Amplitudes of the spontaneous currents were measured with respect to the final level reached after complete decay of the current to a quiet base line. The data are presented here as mean ± S.D.

**RESULTS**

**Spontaneous Currents and Light Responses in norpAP24**—It is well known that light responses in severe norpA hypomorphs have extremely slow kinetics, with responses to brief flashes continuing for several minutes (14) because of the generation of quantum bumps with exceptionally long latencies (15, 16). This behavior is believed to reflect a general principle of G-protein-coupled signaling, namely that the effector enzyme (in this case PLC) also functions as an obligatory GTPase-activating protein required for inactivation of the activated GTP-bound Gα subunit. In WT flies the density of G-protein and PLC (approximately 100 copies/microvillus) is such that diffusional encounters of G-proteins with PLC occur within milliseconds, resulting in rapid activation of PLC swiftly followed by GTPase activity and inactivation. However, when PLC levels are very low (<1 copy/microvillus in severe norpA hypomorphs), activated G-protein α subunits can remain in the active GTP-bound form and diffuse for many seconds or even minutes before finally encountering a PLC molecule, resulting in a greatly delayed cycle of activation and inactivation (15, 16). Consequently, on establishment of the whole-cell recording configuration there is an ongoing barrage of noisy inward current because of the summation of bumps with extremely long latencies generated by previous illumination. Depending on the severity of the allele, this activity decays in the dark during several seconds or minutes after which responses can be elicited from a quiet base line (15–17).

Although it is considered as a null mutation, we wondered whether a similar behavior might also be observed in norpAP24. Indeed, in almost all whole-cell recordings from dissociated norpAP24 photoreceptors we detected a small ~15 pA noisy inward current on establishment of the whole-cell configuration. This current appeared to be mediated by the light-sensitive channels, because it was blocked by Lat3+ (Fig. 1), which is known to specifically block TRP channels in these cells (26, 27). As in other norpA hypomorphs, the noisy current gradually subsided and in favorable recordings eventually revealed isolated ~1–2 pA events (1.4 ± 0.1 pA, n = 5 cells; mean ± S.D.) and finally, after ~20 min, a quiet base line. At this point bright flashes delivered to the cells induced tiny (<2 pA) light responses consisting of little more than a train of miniature “quantum bumps,” again ~1–2 pA in amplitude and lasting for several minutes (Figs. 1 and 5). Because they would be buried in the noise, such responses could be detected only after the complete decay of the spontaneous current and even then only in recordings with excellent signal-noise ratios. Under standard recording conditions, we never detected any response to even saturating flashes (corresponding to >106 rhodopsin photoisomerizations) in norpAP24 when they were delivered before the decay of the initial spontaneous activity.

**Enhancement of Sensitivity by ATP Depletion**—We recently discovered that sensitivity to light in less severe norpA alleles...
such as norpA<sup>P12</sup> and norpA<sup>P116</sup> could be greatly enhanced by the omission of ATP from the recording pipette. This facilitation was effectively mimicked by the rdgA mutation encoding DAG kinase, suggesting that DGK normally suppresses bump amplitudes in norpA<sup>P24</sup> by metabolizing DAG (17). We performed similar experiments to see whether ATP depletion could also enhance sensitivity in norpA<sup>P24</sup>. In recordings made with no ATP in the electrode, the spontaneous activity seen on establishment of the whole-cell configuration in norpA<sup>P24</sup> was now restored to near WT levels (~7 pA) but terminated more slowly (Figs. 2 and 5). Once a quiet base line had been reached, it was maintained indefinitely in the dark (~30') with no sign of spontaneous activation of the light-sensitive channels as happens in WT flies recorded without nucleotide additives (8, 28). Strikingly, sensitivity to light was greatly enhanced with maximum responses up to ~75 pA, and large quantum bumps, similar to the spontaneously occurring bumps during the latter stages of the decay, could be elicited by dimmer flashes (Fig. 2).

Facilitation in norpA<sup>A</sup>, rdgA<sup>A</sup> Double Mutants—To test whether the rescue of light responses in norpA<sup>P24</sup> by ATP depletion could be explained by the failure of DGK as reported in other norpA hypomorphs (17), we generated a norpA<sup>P24</sup>, rdgA<sup>A</sup> double mutant using the most severe allele, rdgA<sup>A</sup> (18). As will be described in detail elsewhere, 2 retinal degeneration was effectively rescued in such double mutants. In norpA<sup>P24</sup>, rdgA<sup>A</sup>, even with standard electrode solution containing nucleotide additives, the constitutive inward currents recorded immediately on establishing the whole-cell configuration were greatly increased (mean 145 ± 31 pA; n = 19) compared with norpA<sup>P24</sup>. Once again, however, the spontaneous currents slowly decayed, reaching a quiet base line after ~20 min. Toward the end of this decay, large (~8 pA) slowly terminating quantum bumps could be resolved, very similar in both amplitude and kinetics to those seen in recordings from norpA<sup>P24</sup> without ATP (Figs. 3 and 5). Sensitivity to light was also massively enhanced on the rdgA background, and even before the decay of the spontaneous current, bright flashes elicited responses up to ~200 pA in amplitude superimposed on the constitutive current. After decay to base line, large bumps could be elicited by dimmer flashes, whereas bright flashes elicited responses of up to ~400 pA that decayed with the slow kinetics that are characteristic of norpA<sup>P24</sup> (Fig. 3). Overall, the rdgA mutation therefore closely mimics the effect of depletion of ATP, and we conclude that impairment of DGK function is sufficient to account for the effect of ATP depletion in enhancing the spontaneous currents, increasing bump amplitude, and rescuing the macroscopic response to light in norpA<sup>P24</sup>. In fact, the overall rescue of sensitivity was even more pronounced in norpA<sup>P24</sup>, rdgA<sup>A</sup>, probably indicating that residual endogenous ATP can support limited DGK function in recordings made without ATP.

Compared with recordings from norpA<sup>P24</sup> made with ATP, the overall facilitation in norpA<sup>P24</sup>, rdgA<sup>A</sup> (estimated by comparing the integrals of light-induced currents) was in excess of 2000-fold (2215 ± 884-fold, n = 3). An ~50-fold increase can be attributed to the increase in the size of the bump integral current (Fig. 5), the rest presumably represents an increase in QE. In fact QE in norpA<sup>P24</sup>, rdgA<sup>A</sup> was now estimated to be reduced only ~30-fold with respect to that seen in WT flies. This comparison highlights both the critical role of DGK, presumably in regulating the supply of DAG required for excitation, and also the considerable potential sensitivity to light in the supposedly null PLC mutant norpA<sup>P24</sup>.

Light Responses in norpA<sup>P24</sup> Are because of Residual PLC Activity—The spontaneous currents and light responses revealed in norpA<sup>P24</sup> could represent residual PLC activity or could be evidence of an alternate phototransduction pathway. To test whether the residual responses in norpA<sup>P24</sup> and

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<sup>2</sup> R. C. Hardie and P. Raghu, unpublished data.
Fig. 4. Gaq1 mutation abolishes spontaneous currents and reduces sensitivity in norpA24. A, responses in norpA24,Gaq11 to a flash containing 106 WT effective photons. This cell was the most sensitive of all norpA24,Gaq11 double mutants tested, and several cells (5 of 11) gave no detectable response. B, responses to flashes containing \(4 \times 10^4\) effective photons in the same norpA24,Gaq1 cell, and also in norpA24,rdgA1,Gaq1. Large quantum bumps with deactivation defects are clearly resolved on the rdgA background, whereas only two or three \(~1-2\-pA\) bumps are seen in norpA24,Gaq11. In both norpA24, Gaq1 and norpA24,rdgA1,Gaq1, the spontaneous current was completely abolished.

norpA24,rdgA1 were mediated by the same PLC-specific Gq protein, we generated norpA24,Gaq1 double mutants and norpA24,rdgA1,Gaq1 triple mutants using the severe hypomorphic allele Gaq1 in which levels of the G-protein a subunit are reduced to \(~1\%\) of WT levels (21). The spontaneous currents in these double and triple mutants were completely abolished, indicating that they were strictly dependent on activation of Gq. Responses to light in norpA24,Gaq1 were also greatly reduced compared with norpA24 controls, with at most a few sporadic 1-2-pA bumps to saturating illumination (Fig. 4) and in 5 of 11 cells no detectable response at all. In norpA24,rdgA1,Gaq1, however, the rdgA background again resulted in a massive enhancement of bump amplitude and QE and large slowly terminating quantum bumps very similar to those seen in norpA24,rdgA1 could be elicited by bright flashes from the start of the recording in all cells (Figs. 4 and 5, \(n = 20\)). Nevertheless, compared with norpA24,rdgA1, sensitivity in norpA24,rdgA1,Gaq1 was reduced \(~100\)-fold. These findings show that both the spontaneous current and the light-induced current in norpA24 and norpA24,rdgA1 are dependent on Gq and hence likely to be mediated by its only known target in the photoreceptors, namely PLC. Although in principle one cannot exclude the possibility that Gq might have alternative targets, the striking rescue of the light response in norpA24,rdgA1 and norpA24,rdgA1,Gaq1 (compared with norpA24 and norpA24, Gaq1, respectively) provides strong independent evidence that the residual responses in norpA24 and norpA24,rdgA1,Gaq1 are mediated by PLC, because the rdgA mutation is expected to result in a greater net production of one of the two products of PLC activity, namely DAG.

The question remains whether the PLC responsible for the spontaneous currents and responses in norpA24 represents residual norpA24 protein or an alternative PLC isoform. The Drosophila genome contains only two further identifiable PLC sequences: a PLC\(\gamma\) encoded by the sl (small wing) gene (22) and PLC-21C, which is a second PLC\(\beta\) isoform (29). PLC\(\gamma\) can be excluded, because spontaneous currents and residual light responses similar to those in norpA24 could still be recorded in norpA24,sl double mutants (data not shown). Because there are no mutants of PLC-21C, we attempted to test whether the residual norpA24 protein might be responsible by generating flies with only one copy of norpA24 by crossing norpA24 to a lethal deficiency strain, Df(1)HC244, in which the entire norpA gene and the surrounding genomic region were deleted. The results, however, were inconclusive; the constitutive currents and quantum bump amplitudes in photoreceptors from norpA24,Df(1)HC244 (\(n = 11\)) were not significantly different from homozygous norpA24 controls, but QE did in fact appear to be reduced \(~2\)-fold. Although the latter suggests that residual mutant norpA24 protein is responsible, because the responses were minimal and near the limit of detectability, we treat this result with caution.

**Activation of TRP Channels by Metabolic Inhibitors Is PLC-dependent**—Because any residual light sensitivity in norpA24 still appears to be mediated by PLC, our findings only serve to strengthen the conclusion that Drosophila phototransduction is an obligatory PLC-based pathway. Nevertheless, numerous studies have assumed that norpA24 is a null mutant, and we wondered whether the finding of significant PLC activity in norpA24 had any implications for the conclusions of such studies. For example, we recently demonstrated that PUFAs such as arachidonic acid and LNA could activate the light-sensitive channels. Because LNA was still effective in norpA24, we concluded that activation was downstream of PLC and therefore likely to reflect a direct effect on the channels (6). Subsequently, Minke and colleagues (8) reported that the light-sensitive channels could be activated by mitochondrial inhibitors; since they too found these agents to be effective in norpA24, they also concluded that the agents acted downstream of PLC and that metabolic inhibition might affect the channels directly. These authors also noted that PUFAs can act as mitochondrial uncouplers and proposed that this was responsible for their ability to activate the light-sensitive channels. In view of the importance of these arguments for the mechanism of excitation and because of the significant PLC activity remaining in norpA24, we repeated and extended these experiments.
FIG. 6. Activation of channels by metabolic inhibitors is dependent on PLC activity. A. 0.1 mM DNP applied by puffer pipette (solid lines) rapidly activated an inward current of 200–300 pA in both WT (left) and norpA(G24) photoreceptors recorded soon after establishing the whole-cell configuration (i); in another norpA(G24) cell after decay of the spontaneous current, DNP only amplified the residual quantum bumps (electrode solution containing nucleotide additives) (ii). The small (~5–10 pA) noise-free inward currents are proton currents because of the \( \text{H}^+ \) ionophore action of DNP. \( B \), massive facilitation of light response in norpA(G24) by DNP. \( C \), before application of DNP, a flash (~10^6 photons at arrow) elicited a barely detectable response consisting of sporadic ~1-pA bumps. \( D \), DNP perfusion some minutes later in the dark resulted only in an increase in the size of remaining bumps still being elicited by the earlier flash. Immediately afterward, however, the same light flash elicited a greatly facilitated response (right trace). A, C, DNP applied to norpA(G24-Gaq) failed to activate any current after an ~10-pA ionophore current (left). Right, quantum bumps in norpA(G24-Gaq) generated by an ~10^6-photon flash were greatly enhanced by DNP (above, before DNP application; below, after DNP application). \( D \), DNP applied during an ~150-pA spontaneous current in norpA(G24-Gaq) and recorded shortly afterward establishment of the whole-cell configuration caused a slight suppression of the ongoing current (three superimposed traces). Similar results obtained in four cells (mean suppression, 46 ± 18 pA, \( n = 4 \)).

We first confirmed that mitochondrial inhibitors could activate light-sensitive channels as reported previously (8). Indeed, large currents were reversibly induced within only seconds of application of either 10 \( \mu \)M CCCP or 0.1 mM DNP in WT and norpA(G24) in recordings made soon after establishing the whole-cell configuration (Fig. 6A). Note that both CCCP and DNP induced a noise-free inward current that is probably because of their mode of action as electrogenic proton ionophores, but this current was clearly distinguishable from the larger and noisy currents representing activation of the light-sensitive channels. Because the DNP-induced proton currents were smaller (~5–10 pA) than those induced by CCCP (~50 pA), most experiments were performed using DNP. The light-sensitive channels were invariably activated by DNP or CCCP either with or without ATP in the electrode, although activation was quicker and not indefinitely reversible when no ATP was included. However, when DNP or CCCP was applied after the spontaneous current had first decayed in norpA(G24), we never detected any activation despite repeated and prolonged (>2 min) application (Fig. 6A). In norpA cells recorded with ATP and in cases where isolated spontaneous bumps were still occurring, DNP caused an increase in the size of these bumps. Similarly, light-induced bumps and macroscopic responses were greatly enhanced by DNP, as in recordings made in norpA, rdgA or in norpA without ATP in the electrode (Fig. 6B, cf. Figs. 3 and 4).

In case this behavior was specific to norpA(G24), similar experiments were also performed in other severe norpA alleles, including norpA(Q24), norpA(F24), and norpA(K24). Again DNP and CCCP induced large inward currents superimposed on ongoing spontaneous or light-induced currents, but in no case (\( n = 8 \)) could any channels be activated after the spontaneous currents had decayed to base line, although again responses to light were greatly facilitated (data not shown). In case the failure to activate channels was related to the long recording time required for the spontaneous current to decay (e.g., because of washout of some essential factor), we also applied metabolic inhibitors to norpA(G24-Gaq) (\( n = 4 \)) and norpA(G24-rdgA1-Gaq) (\( n = 3 \)), in which the lack of constitutive current allowed the inhibitors to be tested immediately after establishment of the whole-cell configuration. However, we were never able to detect any activation of the light-sensitive channels by CCCP or DNP at any stage of recording with or without ATP in the electrode, although again the size of the quantum bumps in norpA(G24-Gaq) was greatly increased (Fig. 6C). These results indicate that the activation of light-sensitive channels by metabolic inhibition is strictly dependent on PLC activity and strongly suggest that it reflects enhancement of the spontaneous miniature quantum bumps induced by residual PLC activity, which underlie the spontaneous currents in norpA.

The most obvious interpretation of these results is that metabolic inhibition activates channels via the same mechanism by which light responses and bump amplitudes are enhanced by ATP depletion in norpA, namely the failure of DGK (see Fig. 8). If this is the case, then the mitochondrial inhibitors should be ineffective on rdgA backgrounds where DGK activity is already minimized. Indeed, in recordings from norpA(G24-rdgA1) double mutants, DNP failed to activate any inward current, whether applied during or after the decay of the spontaneous current (\( n = 4 \), Fig. 6D). In fact, there was typically a slight inhibition of any ongoing spontaneous or light-induced currents. The cause for this was not investigated further, although possible factors might include pH changes or depletion of the GTP required for G-protein activation.

PUFAs Activate Channels Independently of PLC—We performed similar experiments using PUFAs, reasoning that if the ability of PUFAs to activate light-sensitive channels was because of the effect of these acids as mitochondrial uncouplers, then they should also be ineffective in norpA mutants after decay of the spontaneous current. To quantify the data, we applied a subsaturating dose (20 \( \mu \)M) of LNA from a puffer pipette and determined the time taken to elic it a criterion response (10 pA) and also the peak current reached. In marked contrast to the effects of metabolic inhibitors, the potency of PUFAs appeared to be unaffected in norpA mutants. Currents with characteristic high frequency channel noise were elicited by the application of LNA in every photoreceptor tested, both in norpA(G24) after the spontaneous currents had decayed and in norpA(G24-Gaq), which lacks any spontaneous currents (Fig. 7). To confirm that these currents represented the light-sensitive channels, in several cases we tested their current-voltage relationship using voltage ramps, which showed the characteristic outward rectification (Fig. 7F). Compared with WT, the potency of LNA in norpA(G24) backgrounds was if anything slightly enhanced in terms of the time taken to elicit the criterion response, although the maximum current reached was significantly reduced, and the overall waveform of the currents showed a more gradual development without the accelerating overshooting phase typically observed in WT photoreceptors. This overshooting phase could represent positive feedback by Ca\(^{2+}\)-dependent activation of PLC or possibly an additional effect of LNA as a mitochondrial inhibitor or inhibitor of DGK, but it was not explored further. For the present argument, the
Fig. 7. Activation of channels by linolenic acid. 20 μM LNA (bars) applied to a WT photoreceptor (A) and a norpAP24 photoreceptor after complete decay of the spontaneous current (B) in both cases elicited a current with a characteristic high frequency channel noise. Although the final current in WT was larger, the response in norpAP24 developed more quickly (see data not shown). Similarly, channels in norpAP24,sl double mutants were also reliably activated by LNA after decay of the constitutive currents in all cells tested (n = 4) with undiminished potency (Fig. 7). These results indicate that activation of the light-sensitive channels by LNA is not mediated indirectly by activation of PLC.

Discussed, final, if activation of the PLC-21C (or any other undiscov- ered PLC isoform) were responsible for the effects of PUFAs, then we would predict that, like the spontaneous activity and residual response to light in norpAP24, the effects of PUFAs should be greatly enhanced on an rdgA background or in recordings made without ATP. However, we found no significant difference in either latency or the magnitude of the currents activated by LNA in norpAP24,rdgA1 or norpAP24,rdgA1;Gaq1 compared with norpAP24 or norpAP24,Gaq1 controls or in norpAP24 recorded with or without ATP in the electrode (Fig. 7).

In conclusion, activation of the light-sensitive channels by PUFAs cannot readily be explained either by their action as mitochondrial uncouplers or by their reported ability to activate PLC in some systems. This suggests that, as originally proposed (6), PUFAs may act directly on the light-sensitive channels or their lipid environment.

**Discussion**

In this study we found that the supposedly null PLC mutant, norpAP24, has detectable spontaneous and light-activated currents. Although this might have indicated an alternative parallel transduction pathway, further experiments strongly supported the essential role of PLC. Thus, the residual responses still depend on the same PLC-specific G protein and were massively enhanced in norpA,rdgA double mutants in which metabolism of one of the products of PLC activity, i.e. DAG, is blocked. This massive facilitation underscores other recent evidence indicating that DAG or a downstream metabolite is the essential messenger of excitation in *Drosophila* phototransduction (6, 7, 17).

There is still uncertainty, however, as to the identity of the PLC responsible for the responses in norpAP24. The molecular lesion in norpAP24, which has been identified as a 28-bp deletion resulting in a frameshift and a premature stop codon, eliminates the domains believed to be essential for G-protein interaction and renders protein levels undetectable on Western blots (14). Although one would thus expect any residual protein to be non-functional, the mutation leaves the catalytic site still intact so that the possibility of a low efficiency of activation cannot be completely excluded. For technical reasons, we were unable to convincingly demonstrate a further loss of sensitivity in norpAP24/Df(1) flies with only one copy of the norpAP24 gene, and so the possibility also remains that an alternative PLC isoform mediates the residual responses. Of the two remaining PLC isoforms known in the *Drosophila* genome, we could rule out the PLCγ encoded by the sl gene (22), leaving PLC-21C (29) as the only other obvious candidate.

**Mechanism of Activation by Metabolic Stress**—When nucleotide additives are omitted from the electrode solution, the light-sensitive channels in *Drosophila* photoreceptors open spontaneously, generating a so-called rundown current after several minutes of whole-cell recording (8, 28). The underlying cause and its relevance to the physiological mechanism of activation had remained obscure, but the present results now provide an explanation. Although the ability of metabolic inhibitors to activate TRP and TRPL channels in norpAP24 was taken as evidence that the mechanism must be downstream of PLC (8), we found that metabolic inhibitors failed to activate...
channels in the absence of spontaneous currents caused by ongoing residual PLC activity. Both here and elsewhere (17), we found that quantum bump amplitude was reduced in norpA mutants but could be greatly increased by depleting ATP. Hence, metabolic inhibitors can be expected to increase the amplitude of the spontaneous bumps responsible for the constitutive currents in norpA, giving the appearance of the activation of a large inward current. Because the rdgA mutation and ATP depletion have similar effects on bump amplitude, it seems likely that the mechanism of channel activation by metabolic inhibition in these cases is by the impairment of DGK (Fig. 8). Failure of DGK could have at least two consequences: 1) build-up of excess DAG and 2) impaired ability to resynthesize PIP$_2$ (Fig. 8). As argued elsewhere, excess DAG seems to be responsible for enhancing the bump amplitude in norpA (17). We therefore attribute the ability of metabolic inhibitors to activate an inward current in norpA mutants to the impairment of DGK and consequent amplification of the small bumps underlying the spontaneous current by increased levels of DAG. In WT flies, basal PLC activity is presumably sufficient to generate enough DAG to cause activation of the channels if DGK function is compromised, and indeed in the single rdgA mutant, we have found previously that the light-sensitive channels are always constitutively active (7).

Although impairment of DGK seems a sufficient explanation for activation of the channels under the conditions of these experiments, metabolic inhibition will have many other consequences that might also affect the phototransduction cascade. For example, in the long term PIP$_2$ is likely to be depleted, because apart from DGK there are at least three high-energy phosphate-dependent steps required for PIP$_2$ resynthesis (Fig. 8). Furthermore, rhodopsin, arrestin, the PDZ domain-scaffold-ing molecule INAD, and the TRP channels are all phosphorylated by PI kinase and PIP kinase (genes yet to be identified). With other recent evidence (6, 7, 17), the results suggest that under some conditions PIP$_2$ depletion may contribute to TRP or TRPL channel activation (20, 37), and this result might be explained for example if the phosphorylation state of the channels or related proteins determines whether or not PIP$_2$ depletion can activate the channels.

**Activation by PUFAs**—In marked contrast to metabolic inhibitors, PUFAs such as LNA invariably activated the light-sensitive channels in all genetic backgrounds tested, regardless of the presence or absence of PLC and whether or not there was an ongoing spontaneous current. The effects of PUFAs are hence distinct from those of metabolic inhibitors and cannot be explained by their action as mitochondrial uncouplers as suggested previously (3, 8). Neither can their action be readily accounted for by stimulation of PLC, as has been proposed also (20). Thus, the potency of LNA was diminished in mutants of the only PLC isofrom in *Drosophila* genome and was also unaffected in norpA$^{rdgA}$, although the rdgA mutation massively facilitates the activation of the residual PLC activity in norpA$^{rdgA}$. The proposal that PUFAs act directly on the channels (6) or their lipid environment thus still seems the most likely explanation for their effect. Whether PUFAs are the endogenous excitatory messengers, presumably released from DAG by DAG lipase (Fig. 8), or whether they mimic the effect of DAG, for example, remains to be determined. Key questions that need to be answered in this respect include: whether a DAG lipase (which has yet to be cloned in any eukaryote) is expressed in *Drosophila* photoreceptors; if so, whether this lipase is required for phototransduction; and whether there are lipid binding domains on the TRP and TRPL channels or associated proteins.

**Concluding Remarks**—In summary, although we have shown that even a supposedly null norpA mutant can respond to light, our results only strengthen the conclusion that activation of PLC is absolutely required for phototransduction in *Drosophila*. As also discussed elsewhere (17), the striking rescue of the light response in norpA by the rdgA mutation highlights the essential role of DGK in regulating the supply of the excitation messenger and provides compelling support for the proposal that DAG rather than inositol 1,4,5-trisphosphate is the critical product of PIP$_2$ hydrolysis by PLC in *Drosophila* phototransduction. Finally, we propose that this critical role of DGK means that inhibition of this enzyme is the primary although not necessarily exclusive mechanism by which metabolic inhibition can activate the light-sensitive TRP and TRPL channels. Given the widespread distribution of TRP channels in mammalian tissues and the emerging view that many of these may also be regulated by lipid messengers such as DAG and arachidonic acid (10, 38), it will be interesting to see whether this proves to be a more general mechanism of hypoxia-induced Ca$^{2+}$ influx and resultant cytotoxicity.

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3 R. C. Hardie, unpublished data.
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Rescue of Light Responses in the Drosophila 'Null' Phospholipase C Mutant, norpA
P24, by the Diacylglycerol Kinase Mutant, rdgA, and by Metabolic Inhibition
Roger C. Hardie, Fernando Martin, Sylwester Chyb and Padinjat Raghu

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