Review

Regulation of *Drosophila* TRPC channels by lipid messengers

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ABSTRACT

The *Drosophila* TRPC channels TRP and TRPL are the founding members of the TRP superfamily of ion channels, which are important components of calcium influx pathways in virtually all cells. The activation of these channels in the context of fly phototransduction is one of the few *in vivo* models for TRPC channel activation and has served as a paradigm for understanding TRPC function. TRP and TRPL are activated by G-protein coupled PIP_2 hydrolysis through a mechanism in which IP_3 receptor mediated calcium release seems dispensable. Recent analysis has provided compelling evidence that one or more PIP_2 generated lipid messengers, as well as PIP_2 itself, are essential for regulating TRP and TRPL activity. Evidence on the role of these lipid elements in regulating TRP and TRPL activity is discussed in this review.

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1. Introduction

The *t*ransient *r*eceptor *p*otential (TRP) family of ion channels was first discovered in *Drosophila* following the analysis of a mutant with a defective light response [1–3]. With the completion of the *Drosophila* genome project, it has become clear that the fly genome contains 13 putative TRP channel genes [4]. These include at least one member of all seven vertebrate TRP subfamilies described to date. Until now nine of these genes have been subjected to some form of genetic analysis and a function has been ascribed for them *in vivo*. Many of these channels appear to work in the context of sensory transduction pathways. However, the only *Drosophila* TRP channels whose mechanism of activation has been studied in any detail are TRP and TRPL. These are two of the three TRPC channels encoded in the fly genome and they function as components of the light-activated conductance in photoreceptors. A limited number of studies have attempted to address the regulation of TRP and TRPL in other tissue such as the antenna [5] and Malphigian tubule [6]. However, these studies are beyond the scope of this review which will address the activation of TRP and TRPL in photoreceptors.

2. TRP channels in *Drosophila* photoreceptors

Three TRPC channels are reported to be expressed in adult *Drosophila* photoreceptors. These include TRP [2,3], TRPL [7] as well as a more recently described member TRPγ [8] whose function is poorly understood. The contribution of these three genes to the light-induced current has been analysed both *in vitro* and *in vivo*. Like other members of the TRP channel superfamily, TRP and TRPL are subunits of putative tetrameric channels and can be broadly characterised as Ca^{2+} permeable non-selective ion channels. Of these, TRP channels dominate the light response and are by far the more Ca^{2+} selective (P_{Ca}:P_{Na} > 50:1 cf. ~5:1 for TRPL). There is little doubt that *in vivo* TRP and TRPL mediate the light response. Both *trp* and *trpl* mutants still respond to light but with characteristically altered ionic selectivity. However, the double mutant *trp*;*trpl* shows no light response indicating that these gene products are required to generate the light-induced conductance [9,10] and that the third TRPC channel (TRPγ) cannot generate any light-activated conductance in isolation. Heterologously expressed TRPL channels have biophysical properties indistinguishable from the native TRPL dependent conductance [11]. Point mutations in the TRP pore region systematically alter the ionic selectivity of the light-induced conductance providing a rigorous demonstration that TRP is a pore forming channel subunit of the native conductance [12]. Nevertheless, there is still some disagreement on the full complement and subunit stoichiometry of the native channels responding to light. When co-expressed heterologously in mammalian cells, a direct physical interaction between TRP and TRPL has been reported [13]. Xu et al. also report the generation of a novel conductance in these cells and suggest that this represents TRP–TRPL heteromultimers. By contrast, a detailed biophysical analysis of wild type, *trp* and *trpl* mutant photoreceptors suggested that the light-induced current is composed of two separate currents with the properties of the *trp* and *trpl* dependent channels determined using the

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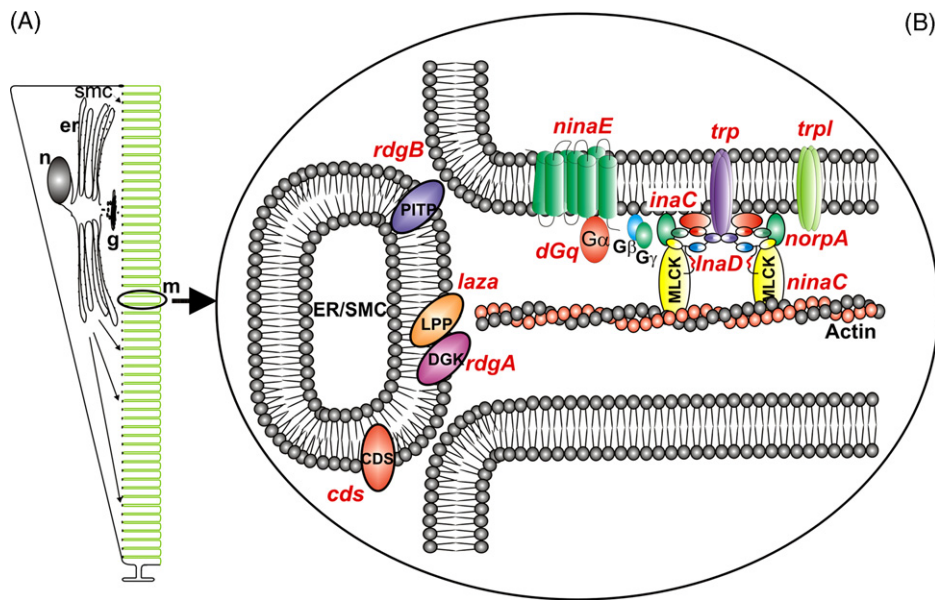*E-mail address:* raghu.padinjat@bbsrc.ac.uk (P. Raghu).

(A)   (B)

Fig. 1. (A) Organization of transduction in *Drosophila* photoreceptors: Longitudinal schematic view of a single photoreceptor showing the rhabdomere (green) composed of finger like projections called microvilli (m). The cell body contained housekeeping organelles such as the nucleus (n), Golgi apparatus (g), endoplasmic reticulum (er). The submicrovillar cisternae (SMC), are specialised smooth endoplasmic reticulum compartments located at the base of the microvilli and contains key transduction components. (B) An expanded view of a microvillus and associated SMC. The localization of known transduction components is shown. Mutants in each component where available are shown in italics. Transduction components and mutants in these are described in the text. PITP, phosphatidylinositol transfer protein; G_α, G_β and G_γ are the α, β and γ subunit of the heterotrimeric G-protein.

respective mutants [10]. Reuss et al. found that the total light-induced conductance could be explained quantitatively as the sum of TRP and TRPL dependent currents arguing against the existence of heteromultimers. The role, if any of TRPγ in phototransduction is unclear, as no mutants have yet been described.

3. Biochemical mechanisms underlying TRP and TRPL activation

The activation of TRP and TRPL in *Drosophila* photoreceptors is mediated by a G-protein coupled signalling cascade where the absorption of a photon by rhodopsin culminates in an inward current and calcium influx into photoreceptors [14]. The transduction cascade that links rhodopsin to activation of TRP channels requires

the activity of the G-protein Gq and phospholipase Cβ (PLC) [Fig. 1]. Severe hypomorphs of Gq (dGq^1) show greatly reduced sensitivity [15] and near null mutants in PLC (*norpA*) show essentially no response to light [16]. Although G-protein coupled phosphatidylinositol 4,5-bisphosphate (PIP$_2$) hydrolysis is an essential element of the transduction mechanism, the biochemical messenger produced that results in TRP and TRPL activation remains controversial. It was originally hypothesized that the (inositol 1,4,5-trisphosphate) IP$_3$ produced by PIP$_2$ hydrolysis would deplete internal calcium stores and that TRP and TRPL in photoreceptors would be activated as a consequence of Ca^{2+} release [17]. However, a number of lines of evidence indicate that this is unlikely to be the case. Release of caged calcium [18] or caged IP$_3$ [19] or thapsigargin [20,21] all fail to activate photoreceptor TRP and TRPL channels *in situ*. Further, a null
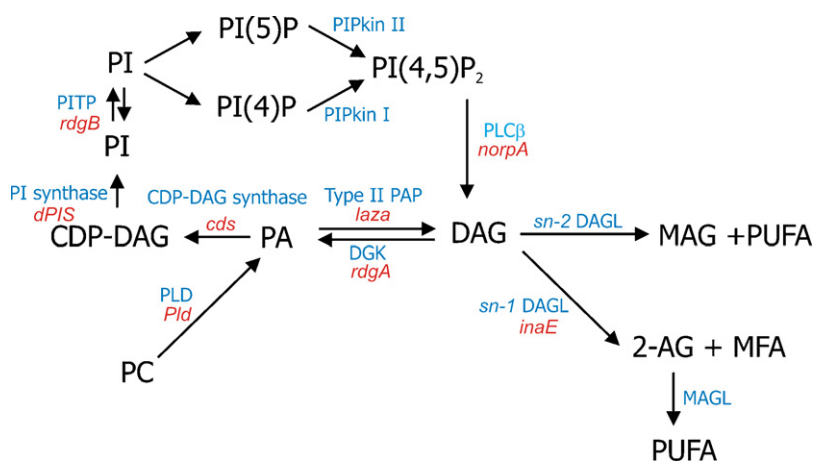

Fig. 2. The key lipid intermediates during G-protein coupled phosphoinositide turnover in *Drosophila* photoreceptors: PIP$_2$ (phosphatidylinositol 4,5-bisphosphate), DAG (diacylglycerol), PA (phosphatidic acid), CDP-DAG (cytidine diphosphosphate diacylglycerol), PI (phosphatidylinositol), PI(4)P (phosphatidylinositol 4-phosphate), PI(5)P (phosphatidylinositol 5-phosphate), MAG (monoacylglycerol), PUFA (polyunsaturated fatty acids), 2-AG (2-arachidonoyl-glycerol), and PC (phosphatidylcholine). Enzymes involved in the turnover of these lipid intermediates are shown in blue. Where available, *Drosophila* mutants of these enzymes are shown in red italics. PLC (phospholipase Cβ), DAGL (diacylglycerol lipase), MAGL (monoacylglycerol lipase), DGK (diacylglycerol kinase), Type II PAP (Type II phosphatidic acid phosphatase), PITP (phosphatidylinositol transfer protein), PIPkin I (PI(4)P-5 kinase), PIPkin II (PI(5)P-4 kinase), and PLD (phospholipase D).

mutant in the only IP$_3$ receptor in the *Drosophila* genome showed normal phototransduction [22,23]. Thus IP$_3$ receptor mediated calcium release appears dispensable for TRP and TRPL activation.

These, at the time unexpected findings, prompted a reconsideration of the biochemical consequences of PLC activity that might be responsible for TRP and TRPL activation. In particular, attention has increasingly focussed on the lipid products of PIP$_2$ hydrolysis.

4. Diacylglycerol kinase is a key regulator of TRP and TRPL during phototransduction

In photoreceptors the major genetic evidence implicating PIP$_2$ derived lipids in activating TRP and TRPL has come from analysis of mutants in the *rdgA* gene that encodes an eye-enriched diacylglycerol kinase (DGK) activity [24] (Fig. 2). In most eukaryotic cells, DAG is phosphorylated by DGK to generate phosphatidic acid (PA) (reviewed in [25]). The lack of this DGK activity results in severe retinal degeneration in *rdgA* photoreceptors [26]. Although originally reported to be light independent and therefore presumably unrelated to phototransduction [24], a recent detailed analysis has shown that degeneration in *rdgA* can be accelerated by light and suppressed by loss-of-function mutants in Gq and PLC [27]; i.e. the key elements required for PIP$_2$ hydrolysis during phototransduction. These new findings suggest that the retinal degeneration phenotype of *rdgA* is related to an imbalance of DGK and PLC activity during phototransduction.

Whole-cell patch-clamp recordings from *rdgA* photoreceptors show constitutively active TRP and TRPL channels in the absence of light [28]. The constitutive activity of the TRP channels in particular appears to be required for the severe degeneration phenotype since *rdgA*;*trp* (but not *rdgA*;*trpl*) double mutants show rescue of degeneration [28]. Unlike severe alleles of *rdgA*, *rdgA*;*trp* double mutants can respond to light; the responses of TRPL channels monitored in these double mutants are larger than control cells and show an increase in the time taken for deactivation. These findings indicate a role for DGK in deactivating the light response, consistent perhaps with a role for DAG in excitation [28]. This amplification and response termination defect in *rdgA* has also been observed at the level of the quantum bump, which represents the unitary response to light [29]. These quantum bumps are normally ca. 10 pA in amplitude but are reduced to only ~2 pA in hypomorphic alleles of Gq and PLC (*norpA*) [30]. However, in the double mutants *rdgA*;*Gq* and *norpA*,*rdgA* quantum bump amplitudes are restored to near wild type values. Together these analyses strongly suggest that *in vivo*, the conversion of DAG to PA is a critical step in regulation of TRP and TRPL activity during the light response.

5. The DAG/PA balance during PIP$_2$ turnover impacts on TRP channel activation

The need to accurately regulate the DAG/PA balance in photoreceptors has been highlighted by two recent studies describing a novel gene that might play an important role in this process [31,32]. This gene encodes a *Drosophila* ortholog of Type II PA phosphatase (lipid phosphate phosphohydrolase, LPP), that could biochemically reverse the activity of DGK, i.e. dephosphorylate PA to generate DAG (Fig. 2). The *Drosophila* genome encodes seven genes that could encode this activity; five are expressed in adult photoreceptors and one of these *lazaro* (*laza*) is enriched in the eye, usually an indication of a role in photoreceptor function. Direct biochemical measurements revealed that loss-of-function mutants in *laza* show light-dependent accumulation of PA relative to wild-type controls indicating that this enzyme plays a non-redundant role in regulating PA levels during phototransduction [31]. The inability to regulate PA levels in *laza* mutants has functional consequences

for photoreceptors, i.e. they undergo light dependent degeneration [31]. It has also been reported that the lack of *laza* activity results in a reduction in the maximum amplitude of the electrical response to light and that responses terminate faster than in wild type flies under similar conditions [32] although an independent analysis of *laza*^{22}, an independent mutant allele, [31] using both ERG and whole-cell recording (Raghu, P., Hardie, R.C., unpublished) could not confirm this phenotype.

Perhaps the most well understood phenotype of *laza* with respect to the regulation of TRP and TRPL activity is its interaction with *rdgA*. Garcia-Murillas et al. [31] showed that overexpression of *laza*, whilst having no effect by itself was able to enhance degeneration in *rdgA*. This enhancement was associated with a further reduction in PA levels that were already lower in *rdgA* compared to wild type photoreceptors. This study also showed that *laza* loss-of-function mutants were able to reduce the rate of degeneration of *rdgA* in a dose dependent manner. The net biochemical outcome of *rdgA*/*laza* activity also has important consequences for TRP channel activation. Garcia-Murillas et al. [31] showed that the deactivation defect of *rdgA* could be modulated by *laza* activity. Overexpression of *laza* enhanced the deactivation defect in *rdgA* while this defect was reduced in *rdgA*;*laza* double mutants. Together all of these findings suggest that during phototransduction *rdgA* and *laza* function as a kinase/phosphatase pair that regulates the balance of DAG and PA and that this balance has important consequences for TRP and TRPL activity and cell maintenance.

6. Biochemical consequences of DGK deficiency

Although the results discussed above strongly suggest that the DAG/PA balance plays a key role in activation and deactivation of the light response, the biochemical consequences of DGK deficiency that result in these effects remain to be resolved. This is important because the identity of the altered lipid species that cause the increased channel activity in *rdgA* is likely to be critical in defining the identity of the messenger of excitation and the mechanism by which TRP channel activity is regulated. A number of biochemical consequences are possible and will be considered below.

6.1. Diacylglycerol

While the finding that loss of DGK results in severe retinal degeneration might suggest that excessive activation of PKC (protein kinase C) could underlie the *rdgA* phenotype, the phenotypes of *inaC* [33] (Fig. 1), that encodes a major source of PKC activity in photoreceptors is not consistent with this model. Two groups have reported that loss-of-function mutants in *inaC* are unable to suppress degeneration in *rdgA* [24,28] and *inaC* mutants themselves show clear defects in response termination, whilst activation appears normal [34]. Indeed, the deactivation defect in *inaC* is contrary to the proposal that the phenotypes of *rdgA* are mediated by excessive PKC activity. However, over the years, DAG binding proteins other than PKC have been described [35] and it is possible that the effects of DAG may be mediated by direct interaction with TRP and TRPL channels or indirectly via another protein. Alternatively channel activity might be regulated by a metabolite of DAG such as polyunsaturated fatty acids (PUFAs) [see below].

The suggestion that TRP and TRPL might be directly activated by DAG is an attractively simple hypothesis that appears generally consistent with the effects of *rdgA* and *laza* mutations and overexpression described above. It also sits comfortably with the widely accepted role of DAG as a key activator of a subset of mammalian TRPC channels (TRPC2, 3, 6, 7) (reviewed in [36]) [Albert et al., this issue; Beech et al., this issue]. However, the proposal that DAG activates TRP and TRPL in photoreceptors is compromised by the lack

of convincing data showing activation of the channels by exogenous DAG. Attempts to activate native TRP or TRPL channels by DAG or analogues applied to photoreceptors, either from the bath or via the patch pipette have been unsuccessful at concentrations up to 100 μM even in *rdgA* mutant backgrounds (Hardie, R.C., unpublished results). Similarly, heterologously expressed TRPL channels in *Drosophila* S2 cells have proved insensitive to exogenous DAG (Hardie, R.C., Raghu, P., unpublished results). Only one study has reported activation by DAG of TRPL channels expressed in *Sf9* cells, but this activation was weak, and reported to be blocked by the PLC inhibitor U73122 leading the authors to question whether the effect was direct [37].

6.2. Polyunsaturated fatty acids

Although DAG itself appears at best to be a reluctant activator of TRP and TRPL, strikingly, the channels can be robustly activated by potential DAG metabolites, namely a variety of poly- and mono-unsaturated fatty acids, including arachidonic (AA), linolenic, linoleic and oleic acids at concentrations as low as ~2 μM. Both TRP and TRPL channels are reliably activated by PUFAs *in situ*, even in *norpA*;*dGq* double mutants [38,39] and heterologously expressed TRPL channels are activated, even in inside-out patches with an EC_{50} (for linolenic acid) of ~10 μM [37,38]. TRPL channels can also be activated by non-metabolizable analogues of AA (ETI or ETYA), which is generally taken as evidence that action is direct rather than via one of the many potential PUFA metabolites. The suggestion that the effects of PUFAs might be mediated indirectly by their role as mitochondrial uncouplers [40] has since been convincingly discounted [39]. In the original report [38], evidence for endogenous production of PUFAs was provided by finding that channels could also be activated *in vivo* by inhibitors of lipoxygenase (LOX)—a class of enzyme which metabolises PUFAs. However, since then it has been recognised that the *Drosophila* genome contains no LOX sequences and the significance of the actions of these inhibitors needs to be reassessed. Interestingly, TRPγ, the third *Drosophila* TRPC channel, was found to have a very similar profile to TRP and TRPL, being sensitively activated by PUFAs and ETYA, whilst being insensitive to DAG [41]. By contrast, the subset of mammalian TRPC channels sensitive to exogenous DAG in general appears not to be activated by PUFAs—although a member of another TRP subfamily (TRPV3) has a remarkably similar pharmacological profile to the *Drosophila* TRPCs [42]. Most recently the activating effect of PUFAs has been shown to be associated with a linearization of the normally strongly outwardly rectifying current–voltage relationship of the TRPL channel [43], which the authors interpret as relief of a voltage dependent open channel block of the channel. A variety of manipulations, which might be predicted to affect the channel-lipid boundary (including osmotic stress, tarantula toxin GMSTx-4 and poly-lysine) resulted in a similar linearization of the *I–V* relation. Together with the "promiscuous" pharmacological lipid profile of channel activation [38], this suggests that PUFAs may act via modulating the lipid environment rather than via a direct ligand interaction with the channel.

6.3. DAG lipase

If PUFAs are the endogenous excitatory messenger in *Drosophila* phototransduction, then clearly the appropriate enzyme(s) required for their generation should be expressed and active in photoreceptors. PUFAs can be directly released from DAG (1-stearoyl-2-arachidonoyl-*sn*-glycerol) by *sn-2* DAG lipase. Alternatively they could be generated in two stages via *sn-1* DAG lipase which generates 2-arachidonoyl-glycerol (2-AG) followed by MAG lipase, which releases AA from 2-AG (Fig. 2). Whilst *sn-2* lipase activity has been well-characterised biochemically in mammalian

cells [44] no gene encoding this activity has yet been identified in any animal.

Two recent studies have analysed mutants in putative DAG lipases that might be involved in generating PUFAs from DAG. Huang et al. reported that mutants in *rbo*, which encodes a gene with a lipase domain show a phototransduction phenotype [45]. The *rbo* mutant shows a reversible loss of photoreceptor activation in ERG recordings following bright illumination. However, the biochemical activity of *rbo* was not directly determined and the reported biochemical phenotypes of *rbo* do not support the idea that it encodes the lipase that converts DAG to PUFAs during phototransduction. First, during illumination DAG levels in *rbo* mutants were reduced relative to wild-type controls [45], a finding contrary to that expected if *rbo* mediates DAG to PUFA conversion. Second, the loss of response was an activity dependent process requiring intense illumination that is inconsistent with a direct role in transduction. Finally, the light dependent reduction in PIP and PIP_2 levels in *rbo* head extracts suggests the loss of responsiveness is probably an indirect effect mediated by loss of PIP_2, an essential substrate for phototransduction.

A more recent study described mutants in a novel gene, *inaE*, which appears to be the *Drosophila* orthologue of the only known mammalian *sn-1* DAG lipase [46]. Recombinant INAE protein was shown to function *in vitro* as a lipase with activity towards 1-stearoyl-2-arachidonoyl-glycerol, preferentially for hydrolyzing the *sn-1* position [47]. Thus this gene encodes a bona fide DAG lipase but not one liberating PUFAs; rather it cleaves DAG to generate a saturated fatty acid and 2-AG (an endocannabinoid). With respect to phototransduction, the basic phenotype of *inaE* hypomorphic mutants described by Leung et al. is qualitatively similar to those of the *trp* loss-of-function mutants: (i) failure to generate a sustained electrical response during ongoing illumination (ii) a refractory period during which the photoreceptors are insensitive to illumination with a second stimulus. Given the resemblance of the phenotype to that of *trp* mutants, it seems likely that the *inaE* gene product affects either the levels of TRP protein or is required for regulating its normal activity during phototransduction. However, the levels of TRP protein in *inaE* have not been reported and the basis of the phenotype is currently unclear.

Before a direct role for INAE in TRP/TRPL channel activation can be accepted, a number of issues need to addressed: (i) the INAE *sn-1* DAG lipase activity generates 2-AG and a saturated fatty acid from DAG, raising the question of whether either of these products activates the channels. Unlike PUFAs, saturated FAs were completely ineffective in activating TRP and TRPL channels *in vivo* or in expression studies [38]. The effects of the other product (2-AG) have yet to be reported. (ii) In principle, an active PUFA species could be released from 2-AG by a further enzyme (e.g. MAG lipase). The *Drosophila* genome encodes several potential candidates for such a gene; it will be important to test whether any of these are active in the photoreceptors and if so whether they are required for excitation. (iii) If fatty acids generated by INAE are involved in TRP channel activation, an immediate prediction is that mutants with reduced levels of INAE would have reduced sensitivity to light as indeed found by Leung et al. [47]; a second prediction is that *inaE* mutants might suppress the constitutive activity and consequent retinal degeneration of *rdgA*. Alternatively, if DAG but *not* a PUFA generated by INAE, is involved in activation, it would be predicted that *inaE* mutants might enhance constitutive activity and degeneration in *rdgA* by restricting the metabolism of DAG. Currently the phenotype of *inaE,rdgA* is unknown. (iv) Using an antibody raised against the INAE protein Leung et al. [47] found that INAE showed an uneven punctate distribution throughout the cell body, with only very occasional punctae detected in the rhabdomere. This immunolocalization pattern seems difficult to reconcile with a direct role for INAE in phototransduction, which is generally believed to take place

within the confines of the microvilli. (v) Other key components of the phototransduction cascade in *Drosophila* are highly enriched in photoreceptors; although INAE is clearly expressed in the photoreceptors, it is also widely expressed at similar levels throughout the head and body; furthermore severe *inaE* mutations are lethal indicating an essential role in addition to any potential involvement in phototransduction.

Finally, there are at least two further obstacles that question the role of DAG and/or PUFAs in excitation. Firstly, the only biochemical determinations of DAG levels in *rdgA* mutants showed no difference between wild type and *rdgA* [49]. Although these measurements were done on total head extracts (rather than eyes), the authors also showed that the amount of DAG in *sine oculis* heads (that lack eyes) was ca. half that in wild type flies, leading them to conclude that the DAG level in *rdgA* eyes was not significantly elevated. It is possible however, that the elevations in DAG levels during light-induced PIP_2 turnover are either small and/or local and below the resolution of these measurements. Interestingly, measurements of PIP_2 hydrolysis indicate that a single photon absorption results in PLC activity at rates sufficient to hydrolyze all the PIP_2 (and probably also the phosphatidylinositol (PI) and PIP reserve) in a single microvillus within less than 1 s [50,51]. A simple calculation based on the PI concentration and dimensions of a microvillus suggests that locally, DAG concentrations within the microvillus can be expected to reach near mM levels. DAG is only poorly soluble in aqueous solutions, and if the channels respond to DAG levels in this concentration range it may be experimentally impossible to apply exogenous DAG at the required concentrations, whilst the more soluble PUFAs may act as surrogate, non-physiological agonists on the same channels.

6.4. Phosphatidic acid

A further predicted consequence of *rdgA* mutations is that levels of PA, the product of DGK could fall and indeed, in contrast to the lack of detectable change in DAG itself in *rdgA*, a reduction in PA has been demonstrated by two independent studies. Inoue et al. [49] showed a dramatic reduction of PA levels in severe alleles of *rdgA*. In a separate and more recent study, PA levels in the hypomorphic *rdgA³* allele were found to be ca. 70% of wild type levels and were further reduced by overexpression of *laza* [31]. This finding suggests that reduction in PA levels might contribute to the *rdgA* phenotype. However, it has been reported that supplementation of PA through the pipette during whole-cell patch-clamp recording failed to suppress the constitutive activity of *rdgA* [28]. More recently Garcia-Murillas et al. tested the effect of elevating PA levels in *rdgA* using a genetic approach. CDP-DAG synthase is the enzyme that condenses PA with CTP to generate CDP-DAG (Fig. 2) and a mutant in the only *Drosophila* gene encoding this activity (*cds¹*) would be expected to show elevated levels of PA [31]. However, they found that *cds¹* enhanced retinal degeneration in *rdgA*. Together these findings suggest that a deficiency of PA *per se* does not underlie the *rdgA* phenotype. However, since both CDS and DGK are required for PI and PIP_2 synthesis (see Fig. 2), these findings raise the possibility that reduced PI/PIP_2 levels may contribute to the phenotypes (see further below).

Since mutants in genes that regulate photoreceptor PA levels such as *rdgA* [24,28], *laza* [31,32] have profound effects on photoreceptor structure and function, there has been interest in the possibility that PA generated by the enzyme phospholipase D (PLD) might play a role in phototransduction, such as providing an alternative excitatory source of DAG. LaLonde et al. [52] reported that photoreceptors from *Pld^null* mutants were less sensitive to light than wild-type controls. PLD clearly cannot mediate phototransduction independently of PLC, and even when PLD was overexpressed Lalonde et al. confirmed that there was no electrophysiological response to light in *norpA* mutants. However, the authors suggested

that constitutive levels of PA generated by PLD might be dephosphorylated to DAG, thereby potentially activating TRP and TRPL channels as suggested for *rdgA* mutants (see above). In support of this they found that overexpression of PLD induced degeneration that was partially rescued in a *trp* mutant [52]. Also, perhaps consistent with this interpretation, Kwon et al. found that retinal degeneration resulting from over expression of PLD was marginally suppressed in a *laza* loss-of-function mutant [32]. However, currently, there is no biochemical data to support the idea that PA generated by PLD can be converted into DAG and no recordings have been made to establish whether PLD overexpression does in fact lead to any constitutive activity. Thus although the reduced sensitivity of *Pld^null* flies to light is intriguing, the role of PLD in phototransduction and especially the function, if any, of the PA it generates remains unclear and requires further investigation.

6.5. Phosphatidylinositol and PIP_2

Since the production of PA by DGK is the first step in the resynthesis of PI and PIP_2 (Fig. 2), levels of these lipids might also change in *rdgA* and contribute to its phenotypes. Although Inoue et al. originally reported that PI levels were not altered in *rdgA* retinae [49], a recent study using mass spectrometry to measure lipid levels found that PI levels were reduced by ca. 40% in *rdgA³* hypomorphs and were further reduced by overexpression of *laza* [31]. These authors also found that the levels of PI synthase transcripts in photoreceptors were correlated with PA levels. Since PIP_2 is produced by the phosphorylation of PI, its levels might also be expected to be reduced in *rdgA* mutants. Although direct biochemical measurements are lacking, recent findings suggest that this is indeed the case and that a reduction of PIP_2 may contribute to the *rdgA* phenotype. Firstly, Hardie et al. [51], using genetically targeted Kir2.1 channels as biosensors for rhabdomeral PIP_2, found that Kir2.1 channel activity was ca. 5–10 fold lower in severe alleles of *rdgA* relative to wild type, indicative of an equivalent reduction in PIP_2. It should however be noted, that these measurements also indicated that *rdgA* photoreceptors still have a substantial basal PLC activity, which should thus continue to generate DAG as in wild type [51]. More recently, Garcia Murillas et al. [31] found that the retinal degeneration phenotype of *rdgA³* was enhanced by a strong hypomorph of *sktl*, which encodes one of four PIP kinases expressed in *Drosophila* photoreceptors. Finally, it is worth noting that PA has been reported as a specific allosteric activator of PI(4)P-5-kinase [53,54]. Whilst there is no direct evidence for such a function in *Drosophila* photoreceptors it can be argued that the lack of PA production in *rdgA* mutants could result in failure to rapidly stimulate resynthesis of PIP_2 by PI(4)P-5-kinase. Together with the finding that *cds¹* enhances rather than suppresses *rdgA* (see above), these results suggest that the phenotype of *rdgA* might be related at least in part to a reduction in the resynthesis of PI and PIP_2.

7. A role for PIP_2 depletion in TRP channel activation?

As substrate for PLC, PIP_2 is essential for phototransduction and it seems that no light response can be elicited in its absence. This is evident for example in the light dependent loss of sensitivity in mutants of key enzymes and transporters required for PIP_2 synthesis including CDP-DAG synthase [58], PI synthase [55] and the PI transfer protein (PITP) encoded by the *rdgB* gene [50,56]. In both *cds* and *rdgB* mutants the near irreversible loss of PIP_2 following illumination has been directly demonstrated *in vivo* by tracking PIP_2 levels during whole-cell recording experiments again using the PIP_2 sensitive ion channel Kir2.1 as an electrophysiological biosensor for rhabdomeral PIP_2 [30,50]. The same approach has been used to demonstrate that the decay of the light response in *trp* mutants

and subsequent refractory period are strictly correlated with (and almost certainly caused by) the near total depletion of PIP$_2$ in the rhabdomere. This is interpreted as a requirement for Ca^{2+} influx via TRP channels to rapidly inhibit PLC. Quantitative analysis indicates that without this negative feedback each absorbed photon results in the depletion of all the PIP$_2$, PIP and PI in a single microvillus within ca. 1 s [50,51].

However, does PIP$_2$ serve only as a substrate for the potential activators of TRP and TRPL channels (e.g. DAG or PUFA) or does it play a more direct and active role? In particular might PIP$_2$ act as an inhibitor of TRP and/or TRPL channels, so that PIP$_2$ depletion actively contributes to their excitation, and by extension contribute to *rdgA* degeneration phenotypes? Evidence for an inhibitory role of PIP$_2$ was first provided by Schilling and co-workers [37] who found that heterologously expressed TRPL channels could be inhibited by exogenous PIP$_2$ in inside-out patches. However, *in vivo*, the complete decay of the response in *trp* mutants is difficult to reconcile with this idea, as it seems contrary to what would be expected if PIP$_2$ depletion by itself were to directly activate the channels. The *trp* decay would, however, be consistent with a role for DAG or its metabolites in excitation (since the substrate for its generation is exhausted). By contrast, TRP channels appear to behave rather differently from TRPL channels in this respect. The Ca^{2+} influx required to inhibit PLC and thus prevent excessive PIP$_2$ hydrolysis can also be blocked by removing extracellular Ca^{2+}; under these conditions flashes of light which deplete a substantial fraction of PIP$_2$ usually result in a failure of the TRP channels to close rapidly after termination of the light flash [50]. A similar failure in response termination is also found in *rdgB* mutants lacking PITP even in the presence of Ca^{2+} [50,56]. Finally, when TRP channels are activated following ATP deprivation they remain active indefinitely, and long after all detectable PIP$_2$ (as monitored by Kir2.1 biosensors) has disappeared from the microvilli [57]. Nevertheless, there are also conditions where PIP$_2$ can be depleted without the persistent activation of TRP channels, including in mutants of *cds* [58] and PI synthase [55], and also under Ca^{2+} free conditions when care is taken to deplete PIP$_2$ gradually with repeated and relatively weak light flashes (Hardie, R.C., unpublished). Whilst these contrasting results have not been fully reconciled, it seems that the conditions where PIP$_2$ depletion fails to activate the TRP channels appear to be those where the total PIP$_2$ hydrolysis and DAG generation, which inevitably accompanies PIP$_2$ depletion in such experiments, is relatively low. In particular, in contrast to *rdgB* mutants, in *cds* and PI synthase mutants, initial PIP$_2$ levels are likely to be low resulting in less DAG production by the stimuli used to deplete PIP$_2$. These observations may indicate that a further event in addition to PIP$_2$ depletion contributes to activation. For example, a hypothesis worth serious consideration is that channels may be activated by simultaneous generation of DAG (or one if its metabolites) and depletion of PIP$_2$ [37].

8. Conclusions and outstanding questions

We have known for over 20 years that PIP$_2$ hydrolysis by PLC is essential for phototransduction in *Drosophila* [16]; however, the resolution of the final mechanism of activation of TRP and TRPL channels has proved a remarkably tough nut to crack. Although numerous converging lines of genetic and physiological evidence now indicate that PIP$_2$ derived lipids, rather than soluble IP$_3$, contribute to TRP and TRPL activation, key outstanding questions remain. The levels of several distinct signalling lipids are altered, or potentially altered as a consequence of PIP$_2$ hydrolysis including DAG, fatty acids, PA and phosphoinositides; the latter are also essential intermediates in the resynthesis of PIP$_2$ whose levels itself of course will fall. As discussed in this review, there is evidence for

the involvement of several of these (including PUFAs, DAG and PIP$_2$ itself), but equally, there are outstanding questions that need to be addressed before any can be firmly accepted, and the principal challenge for the future will be resolving the individual contributions of each of these to activation of TRP channels in photoreceptors. Given the emerging consensus that TRP channels are subject to polymodal regulation, we need also not be surprised if there are in fact two or more critical messengers of excitation such as the simultaneous increase in DAG (or metabolite) and decrease in PIP$_2$.

The particular advantage of *Drosophila* as an experimental model, is its potential for genetic analysis, which is unparalleled amongst higher organisms. In this review we have highlighted examples exploiting studies of genetic interactions between components of the PI cycle (Fig. 2). Similar approaches may be particularly informative in analysing the potential involvement of the large number of DAG and MAG lipase genes encoded in the *Drosophila* genome. However, since the loss (or overexpression) of a single enzyme often results in changes in the levels of lipid metabolites other than the immediate substrate and product of the enzyme in question (e.g. see [31]), it will be of critical importance to be able to measure changes in the levels of individual lipid species in photoreceptor preparations.

It is also essential to test the ability of candidate lipids to activate channels in a physiological preparation. Although photoreceptors are amenable to whole-cell patch-clamp recording, the rhabdomere membrane is not readily accessible for analysis of excised patches (but see Haab et al. [59]) and heterologous expression systems are therefore essential, for example for routine inside-out patch studies. Whilst TRPL channels can be readily expressed and studied in this way [11,19,37,60,61], in general TRP itself has proved difficult to express. Furthermore, none of the few apparently successful studies [13,62,63] have reported channels with biophysical properties matching the native photoreceptor TRP dependent conductance. Hence, the ability to functionally express TRP may be a key step to understanding the mechanism of activation.

If DAG/PIP$_2$ modulate TRP and TRPL, one prediction is that they might bind directly to the channel. None of the TRPC channels contain unequivocal C1 (DAG binding) or PIP$_2$ binding pleckstrin homology (PH) domains. However, there is biochemical evidence for competitive binding of polyphosphoinositides to a calmodulin binding site in the C terminus of some mammalian TRPCs [64], and there is also some evidence for a novel lipid binding domain in the N-terminus [65]. In addition the C-termini of TRPCs do contain polybasic regions which are not dissimilar to PH domains and have been hypothesized to interact with PIP$_2$ [66].

Characterisation and targeted mutagenesis of such domains will be essential for confirming the role of lipids in activating the channel. It is also possible that direct ligand binding may not be an element of TRP and TRP gating. As well as the potential involvement of additional unidentified proteins, it is also possible that small local biophysical/mechanical changes in the membrane in the vicinity of the channels may result in the conformational change resulting in gating [43].

This review has focussed on the roles of lipid messengers in activation of the *Drosophila* TRPC channels. However, resolution of the mechanism of activation of TRP and TRPL in *Drosophila* photoreceptors has broader implications, in particular for the mammalian TRPC channel subfamily members, which are also all activated downstream of PLC but by mechanisms which are still far from clear. Although DAG is widely regarded as an activator of TRPC2, 3, 6 and 7, whether its action is direct or not is unclear, and recent evidence has also implicated PIP$_2$ as an inhibitory factor in the gating of TRPC6 [67,68]. There is even more uncertainty over the mechanism of activation of TRPC4 and TRPC5, but interestingly, again PIP$_2$ has recently been implicated, with both excitatory and inhibitory roles having been reported [69]. More generally still, PIP$_2$ has been

increasingly recognised as a key factor in the activity of most if not all members of the TRP superfamily as well as many other ion channels and transporters (see reviews by Rohacs, Albert et al., this issue [70–73]).

Note added in proof

Very recently, Delgado & Bacigalupo reported that TRP and TRPL channel activity could be activated by the DAG analogue OAG at concentrations as low as 2 microM in inside-out patches excised from the microvilli of dissociated photoreceptors. In principle this would remove one of the main objections to accepting DAG as the excitatory messenger. It should be noted however, that activation in their experiments was slow (up to 60s after application), and it would be premature to conclude that activation was direct.

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