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Light-Induced Opening of the TRP Channel in Isolated Membrane Patches Excised from Photosensitive Microvilli from *Drosophila* Photoreceptors

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Abstract—*Drosophila* phototransduction occurs in light-sensitive microvilli arranged in a longitudinal structure of the photoreceptor, termed the rhabdomere. Rhodopsin (Rh), isomerized by light, couples to G-protein, which activates phospholipase C (PLC), which in turn cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) generating diacylglycerol (DAG), inositol trisphosphate and H^+. This pathway opens the light-dependent channels, transient receptor potential (TRP) and transient receptor potential like (TRPL). PLC and TRP are held together in a protein assembly by the scaffold protein INAD. We report that the channels can be photoactivated in on-cell rhabdomeric patches and in excised patches by DAG. In excised patches, addition of PLC-activator, m-3M3FBS, or G-protein-activator, GTP-γ-S, opened TRP. These reagents were ineffective in PLC-mutant *norpA* and in the presence of PLC inhibitor U17322. However, DAG activated TRP even when PLC was pharmacologically or mutationally suppressed. These observations indicate that PLC, G-protein, and TRP were retained functional in these patches. DAG also activated TRP in the protein kinase C (PKC) mutant, *inaC*, excluding the possibility that PKC could mediate DAG-dependent TRP activation. Labeling diacylglycerol kinase (DGK) by fusion of fluorescent mCherry (mCherry–DGK) indicates that DGK, which returns DAG to dark levels, is highly expressed in the microvilli. In excised patches, TRP channels could be light-activated in the presence of GTP, which is required for G-protein activation. The evidence indicates that the proteins necessary for phototransduction are retained functionally after excision and that DAG is necessary and sufficient for TRP opening. This work opens up unique possibilities for studying, in sub-microscopic native membrane patches, the ubiquitous phosphoinositide signaling pathway and its regulatory mechanisms in unprecedented detail.

Key words: diacylglycerol, *Drosophila*, phospholipase C, photoreceptor, phototransduction, rhabdomere.

INTRODUCTION

Drosophila eyes contain ~800 ommatidia, each enclosing eight photoreceptor cells. Photoreceptors possess a longitudinal structure called rhabdomere, consisting of ~40,000 microvilli housing the phototransduction molecular machinery (Hardie and Juusola, 2015).

Light isomerizes rhodopsin (Rh), which couples to a heterotrimeric Gq-protein that activates phospholipase C (PLC). PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2), generating inositol trisphosphate, diacylglycerol (DAG) and a proton H^+. DAG mediates the activation of two light-dependent channels, TRP (transient receptor potential) and TRPL (TRP-Like) (Raghu et al., 2000), that underlie the photoresponse; the mechanism underlying channel gating remains mysterious. Some phototransduction proteins are assembled together by a protein scaffold, INAD (Tsunoda et al., 1997), namely PLC, protein kinase C (PKC) and TRP, but the molecular organization within microvilli remains undetermined. With electrophysiology, pharmacology, mutational analysis, and fluorescence microscopy we examine the localization of key phototransduction components and their roles in the signaling pathway. Investigating direct roles of PLC pathway components in TRP activation has been difficult, largely because of the uncertainties in interpreting whole-cell electrophysiological recordings from individual photoreceptors, where complex transduction

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Abbreviations: DAG, diacylglycerol; PBS, phosphate-buffered saline; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; TRP, transient receptor potential; TRPL, transient receptor potential like.

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processes within microvilli are only a part of the cell's physiology.

We circumvented this problem by recording directly from sub-microscopic on-cell and excised inside-out rhabdomeral membrane patches (Delgado and Bacigalupo, 2009). This has allowed us to characterize unitary TRP and TRPL currents and obtain firm evidence supporting DAG as their physiological activator (Delgado and Bacigalupo, 2009; Delgado et al., 2014). TRP in excised patches can be opened by direct DAG application and can be subsequently closed by ATP, required by diacylglycerol kinase (DGK) to phosphorylate DAG, returning it to basal levels. Furthermore, the observation that light raises rhabdomeral DAG levels (Delgado et al., 2014) supports the notion that DAG mediates PLC-dependent TRP activation and DAG phosphorylation by DGK terminates the response. There is considerable controversy surrounding the role of DAG in PLC-mediated channel opening, and several phenomena have been proposed to play roles in excitation, including the light-induced reduction in PIP_2 and decrease in pH, causing mechanical gating of the channels (Huang et al., 2010; Hardie and Franze, 2012), and polyunsaturated fatty acids that might be generated from a putative DAG lipase (Chyb et al., 1999; Katz and Minke, 2018).

We previously reported that TRP could be opened by illumination in on-cell patches (Delgado and Bacigalupo, 2009). Here we show that TRP can be activated by light in excised patches in the presence of GTP, illustrating that the phototransduction cascade of reactions, from Rh photoisomerization to channel gating, through G-protein activation and PLC-mediated of PIP_2 catalysis, is retained functionally within isolated sub-microscopic membrane patches. Additionally, we demonstrate, with confocal microscopy, the rhabdomeral localization of DGK.

EXPERIMENTAL PROCEDURES

Flies

Drosophila melanogaster flies were reared in cycles of darkness and dim light at 18 °C. For PLC mutants, we used the severe allele $norpA^{P45}$ (Pearn et al., 1996). For Rh mutants, we used the hypomorphic allele, $ninaE^{P334}$ (Kumar and Ready, 1995). Flies were obtained from Bloomington Stock Center (Bloomington, USA). Flies had one week after eclosion. Eyes were dissected from adult flies and their retinas were mechanically disrupted with a sharp tungsten wire under low divalent cation *Drosophila* Ringer's solution of the following composition (in mM): 120 NaCl, 4 KCl, 1 EGTA, 0.7 $CaCl_2$, 10 HEPES, 2.5 L-proline and 25 sucrose, pH 7.15, pCa 6.2. This procedure yielded a low but sufficient number of ommatidia with partly disaggregated photoreceptors that clearly exposed the apical ends of their rhabdomeres, allowing sealing patch-clamp pipettes for unitary channel current recordings (Delgado and Bacigalupo, 2009; Delgado et al., 2014). The cells were viewed with an Olympus IX70 inverted microscope, using either DIC optics (60× objective) or phase contrast (100× objective, NA 1.25).

Experimental design and statistical analysis

The experiments were designed to record unitary light-activated channel currents from the light-sensitive membrane of *Drosophila* retinal photoreceptors. This study was performed with the patch-clamp technique on both, cell and excised (inside-out) rhabdomeral membranes. The excised patches were stimulated with light and/or with various specific activators of the individual phototransduction enzymes, delivered by pressure from micropipettes. Inhibitors and mutations for these proteins were used to test whether each cascade component stayed functional in the excised patches. We measured the latencies of channel activation. The recorded currents were obtained from at least four membrane patches under each experimental condition tested. The number of experiments is mentioned in the figure legends. We provide the averages of the latencies, expressed as mean ± standard error of the mean.

Electrophysiology

Single-channel currents were recorded with an Axopatch 200B patch-clamp amplifier (Molecular Devices, USA). Pipettes of > 50 MOhm resistance were made of borosilicate glass capillaries with a horizontal puller (Sutter Instruments, USA). Pipettes were filled with low divalent cation Ringer's solution and the same solution was used externally, except for the experiments with light stimulation, in which we used a 10-fold Na^+ gradient to enhance the resolution of the single-channel currents ($Na^+_{pipette}/Na^+_{bath}$ = 120/12, 100 mM Na^+ replaced by equimolar N-methyl-D-glucamine). A double-barreled puffer pipette (theta glass, ~0.5 μm each tip, at ~5 μm from the recording pipette) connected to a computer-controlled picospritzer (custom made) was used to apply the desired solutions onto the patches. Light stimuli were generated with a white light LED. Electrophysiological recordings were digitized by a (Digidata 1440 interface, Molecular Devices, USA) and stored in a PC. pClamp10 software (Molecular Devices) was used for data acquisition and analysis. Recorded currents were sampled at 10 kHz, and low-pass filtered at 500 Hz for analysis. All voltages are expressed as pipette potentials. The recordings were corrected for the shift of the baseline current with the pClamp correction routine.

Chemicals

DAG (1-Oleoyl-2-acetyl-*sn*-glycerol, Calbiochem), was prepared daily from a stock solution in DMSO to final concentration in the divalent cation-free *Drosophila* Ringer's solution; m-3M3FBS (Sigma Products, USA), GTP-γ-S (Merck Millipore, USA), U73122 (Sigma Products). All others were from Merck.

Generation of the fusion protein mCherry–DGK

The rdgA-mCherry flies were made using the *Minos*-mediated integration cassette, which allows genome manipulations (Venken et al., 2011). The MiMIC $rdgA^{MI07776}$ intronic insertion (BL52119) was used to intro-

duce a mCherry coding exon to create a fusion protein that conserves the endogenous distribution and levels of expression of RdgA. Replacement with the protein-tag cassette was made by injecting the Bloomington stock BL52119 with the donor vector containing the mCherry coding sequence in the proper reading frame (plasmid *Drosophila* Genomics Resource Center #1315; Bloomington, USA).

Immunofluorescence

Drosophila heads were fixed in 8% Formaldehyde for two hours under slow shaking, rinsed four times in phosphate-buffered saline (PBS) and permeabilized by incubation in 0.3% Triton X100 in PBS, at room temperature overnight. Later, heads were placed and oriented in a Petri dish, covered with low melting point agarose (Thermo Fisher Scientific, USA) prepared in 4% PBS at 45 °C, and left for 4 h at room temperature. The tissue-embedded in an agarose block was cut in a Vibratome (Pelco, USA) in slices of 400 μm that were kept in PBS. To block non-specific binding sites, the slices were incubated in 2% fetal bovine serum in PBS for 1 h. The primary antibody, mouse anti-TRP (1:200; Hybridoma Bank, USA; catalog number MAb83F6) was diluted in the same blocking solution and incubated overnight at 4 °C under gentle shaking. The slices were then incubated with the secondary antibody mouse Alexa 488 (1:200; Thermo Fisher Scientific, USA; catalog number: A32723) prepared in PBS for 2 h in darkness under gentle shaking, and rinsed twice in PBS. Samples were mounted using Vecta shield mounting media (Vector Laboratories, USA) and visualized with an inverted laser confocal microscope (Carl Zeiss LSM 710 Meta, Germany). The confocal images (0.1 μm slices) were captured and processed using Fiji (Image J, USA) (Schindelin et al., 2012). The final figure montage was performed with Adobe Photoshop (USA), using identical values for contrast and brightness.

RESULTS

We reported previously that the light-dependent channels, TRP and TRPL, opened with light in on-cell rhabdomeral patches, illustrating that Rh, together with these channels and the enzymes mediating their interaction were present and functional in those patches (Delgado and Bacigalupo, 2009). We now investigated whether the key phototransduction proteins, namely Rh, G-protein, PLC, TRP and DGK, were retained and responsive to light stimulation after being excised.

PLC is found in excised patches

To test for the presence of PLC, we puffed internal solution containing the irreversible PLC activator m-3M3FBS (5 μM) onto the patches and evaluated the channel activity in light-insensitive mutants whose TRP channels are closed regardless of illumination: *norpA* (PLC) or *ninaE* (Rh) (Delgado et al., 2014). Applying the drug for about 40 seconds triggered the opening of the channels, as expected since PLC activity leads to DAG production and channel opening (Figs. 1A and 6; $N = 9$ of 10; av. latency 0.9 ± 0.3 s).

To control for the possibility that m-3M3FBS could have acted on the TRP channels directly rather than through PLC, we repeated the experiment in the *norpA* mutant (Fig. 1B; $N = 4$). The drug was without effect, but subsequent addition of DAG activated the channels, indicating that the effect of m-3M3FBS required functional PLC and that the drug does not act directly on the TRP channels.

The G-Protein is found in the excised patches

To assess the presence of G-protein in excised patches, we asked whether activating the G-protein artificially would open TRP channels (in *ninaE*$^-$ mutant background). We used the slowly hydrolysable GTP analog, GTP-γ-S, to activate the G-protein. Fig. 2A shows that 50 μM GTP-γ-S (∼40 s) led to channel opening, consistent with the presence of functional Gq in the patch (Fig. 6. $N = 4$ of 7; av. latency was 47 ± 13 s). We also tested 500 μM, with similar results ($N = 5$ of 7).

A prediction from this observation is that the channels cannot be opened by GTP-γ-S in the absence of functional PLC. Indeed, *norpA* patches stayed silent after introducing GTP-γ-S, but the channels could be then activated by DAG (Fig. 2B; $N = 4$). Similarly, GTP-γ-S failed to induce TRP channel opening when PLC was inhibited by U73122 (5 μM) in *ninaE* flies (Fig. 2C, $N = 7$).

Combined, these experiments demonstrate that the G-protein remains in the patches after excision and strengthen the notion that PLC is also present.

DGK is found in excised patches

A previous immunohistochemistry study concluded that DGK was expressed in the subrhabdomeral cisternae located in the cell body of the photoreceptors and was absent in the rhabdomere (Masai et al., 1997). However, we provided strong electrophysiological evidence that DGK coded by *rdgA* was retained in excised patches and preserved its ability to metabolize DAG (Delgado et al., 2014). Therefore, we revisited this issue with a much more sensitive assay. To this end, we constructed a MiMIC-derived transgenic fly (Venken et al., 2011) that expresses the fluorescent protein mCherry fused with DGK in an otherwise wild-type background. This allowed us to visualize DGK localization in the photoreceptors directly. As shown in transversal and longitudinal retina sections of Fig. 3, mCherry and TRP fluorescence signals were detected only in the rhabdomeres and not in the cell bodies. Importantly, in contrast to *rdgA*$^-$ mutants, flies carrying the fusion protein do not undergo retinal degeneration, indicating that DGK is fully functional. These results ($N = 4$) support our previous conclusion that DGK is expressed in the microvilli, and it is retained and functional in the patches after excision (Delgado et al., 2014).

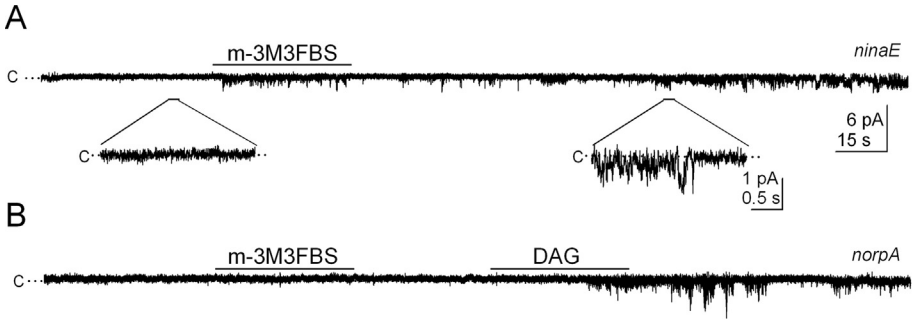

Fig. 1. Phospholipase C activator induces the opening of TRP. (A) PLC activator m-3M3FBS (5 μM) induced a transient TRP activation in an excised patch from a *ninaE; trpl* fly (reduced rhodopsin function and null TRPL); a subsequent DAG application (5 μM) activated the channel (N = 9 of 10; average latency of the activity onset: 0.9 ± 0.3 s, Mean ± S.E.M). (B) Similar protocol with the *norpA* mutant shows no effect of the drug, whereas DAG still opened the channel (N = 4). Pipette potential (Vp): 40 mV.

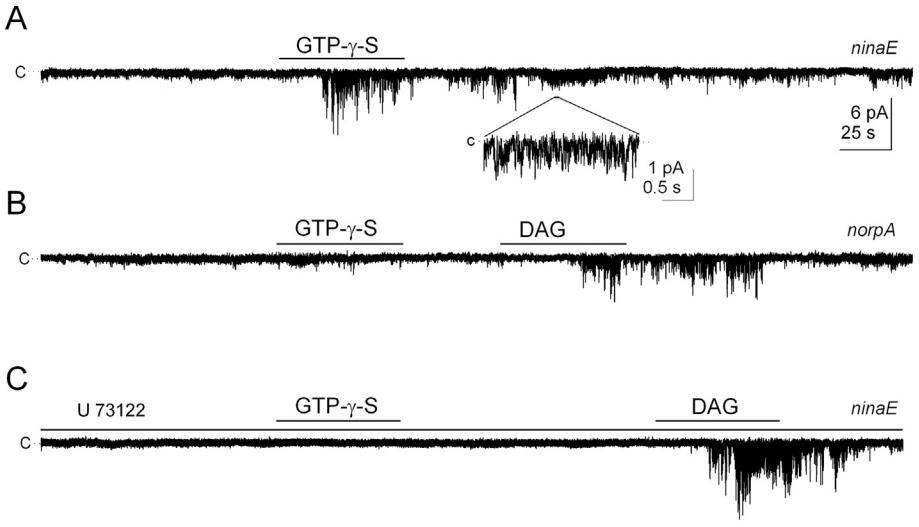

Fig. 2. Protein G activator induces the opening of TRP. (A) G-protein activator GTP-γ-S (50 μM) induced a transient TRP activation in an excised patch from a *ninaE;trpl* fly; a subsequent DAG application activated the channel persistently (N = 4 of 7; 47 ± 13 s). (B) Similar protocol over a *norpA* mutant shows no effect of the drug, whereas the channel was still opened by DAG (N = 4). (C) In the presence of the PLC inhibitor, U73122 (5 μM), GTP-γ-S had no effect, but DAG still activated TRP in a *ninaE* mutant (N = 7). Vp: 40 mV.

DAG-induced opening of TRP channels does not require DAG-dependent PKC

Findings of Delgado et al. (Delgado et al., 2014) and those above illustrate that DAG addition can cause TRP channels to open even in the absence of functional Rh, G-Protein, and PLC, suggesting that the action of DAG occurs at a later step in phototransduction. But how DAG opens channels remains unknown. The transducisome in microvilli includes bound, eye-specific protein kinase C (eye-PKC), which is not required for normal excitation but influences photoreceptor sensitivity and photoresponse deactivation (Ranganathan et al., 1991; Smith et al., 1991; Hardie et al., 1993). Among the phototransduction assembly proteins, only eye-PKC is known to bind DAG, and it requires DAG for its activation (Azzi et al., 1992; Shieh et al., 2002). We sought to determine whether excitation induced by direct application of DAG to excised patches might be mediated by DAG binding to eye-PKC. If this is the case, we expect that DAG would be ineffective in opening TRP channels in the absence of eye-PKC, i.e., in *inaC* null mutants. Fig. 4, however, illustrates that DAG opened TRP in *inaC*$^{-}$ mutants, indicating that DAG binding to eye-PKC is not a required step in DAG-induced excitation (N = 4).

Light can activate TRP channels in isolated rhabdomeral membrane patches

Delgado et al. (2009) showed that TRP channels in on-cell patches could be opened by illumination. In Fig. 5, we show that light can also open TRP channels after the patch has been excised in the presence of 1 mM GTP, which is necessary for G-protein activation. We began by stimulating the ommatidium with light after establishing the gigaseal on the rhabdomere before excision and recorded light-dependent TRP activation (Fig. 5A). We then let the patches dark adapt for at least four minutes before excising them in darkness and applied the same light stimulus (Fig. 5B). 23 (of 60 tested) on-rhabdomeral patches responded to light. Of these 23, nine exhibited photoresponses after excision. As seen in the representative experiment of Fig. 5, we consistently observed that fewer channels opened with light stimulation than with all the other stimuli (GTP-γ-S, m-3M3FBS and DAG). The average latencies of on-cell and excised patches were 6.7 ± 1.4 s and 7.2 ± 2.9 s, respectively, (Fig. 6. N = 23 and 9, respectively).

In all patches under every experimental condition tested the spontaneous activities were highly infrequent compared with the periods of activation (see Figure Legends).

These results demonstrate that the principal proteins of phototransduction are retained in rhabdomeral excised patches in a functional state.

DISCUSSION

In this work we demonstrate that extremely small patches of membrane (~0.12 μm^2; pipettes tip inner diameter ~0.4 μm, as measured by scanning electron microscopy) isolated from the photosensitive organelles of *Drosophila* photoreceptors retain the proteins

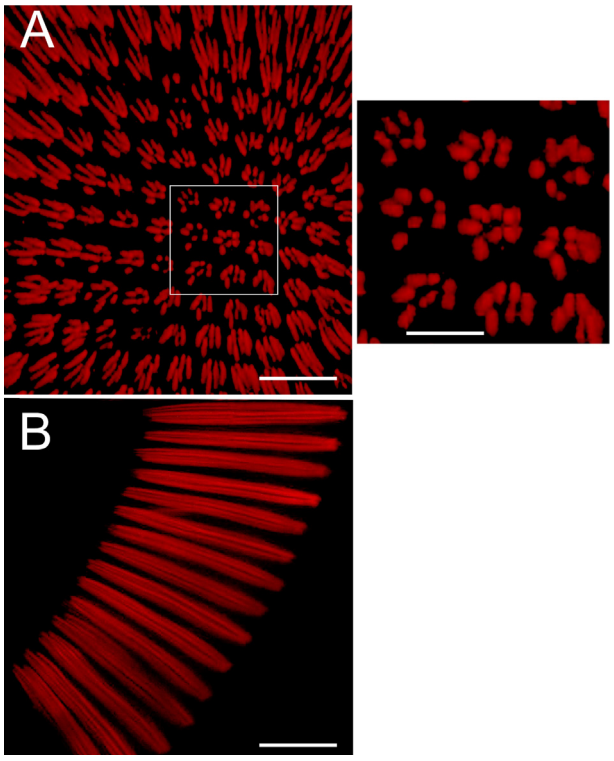

Fig. 3. Diacylglycerol kinase is specifically localized to the rhabdomere. (A) mCherry–DGK fluorescence image of a transversal section of a retina (Bar = 10 μm); a magnified area indicated with a frame is presented at its right side (Bar = 5 μm) (N = 4). (B) Longitudinal section of a retina (Bar: 10 μm) (N = 4).

necessary for phototransduction and respond to light. The evidence supports a model in which phototransduction from photon absorption to channel opening is a highly localized phenomenon.

Previous studies showed that TRP channels in on-cell rhabdomere membrane patches can be activated with light (Delgado and Bacigalupo, 2009). We now show that light activated the TRP channels in the excised patch when GTP was provided; thus Fig. 5 indicates that the cascade components essential for transduction remain functional in the patch after excision. These include integral membrane proteins, Rh, TRP, and TRPL and membrane-associated proteins, G-protein, DGK and PLC. The localization of DGK in the rhabdomere indicated by the powerful mCherry-DGK fusion protein experiment (Fig. 3) confirms prior functional electrophysiological evidence in excised patches showing that activation of this enzyme by adding ATP ended channel activity (Delgado et al., 2014); interestingly, this result was obtained in all tested patches, suggesting that DGK is associated with the phototransduction protein complex. Additionally, the observation that G protein activation by GTP-γ-S occurred in the majority of the tested patches suggests that this membrane-associated protein might also be somehow linked to the signaling complex.

The observation that TRP channels in excised patches can be opened by adding DAG and subsequently closed by adding ATP (Delgado et al., 2014) indicates that DGK-catalyzed phosphorylation of DAG terminates the response, as reported previously (Raghu et al., 2000). The notion that the response ends when DAG is no longer available indicates that this lipid is required for TRP channel opening. Delgado et al (Delgado et al., 2014) further illustrated that DAG is both necessary and sufficient for TRP activation, consistent with a phototransduction model in which DAG is the lipid-soluble messenger linking PLC-catalyzed PIP_2 hydrolysis triggered by light to TRP opening. Importantly and in agreement with our findings, four of the seven members of the TRPC channel family, mammalian homologs of *Drosophila* TRP, namely TRPC 2, 3, 6 and 7, have been reported to be also opened by DAG, (Hofmann et al., 1999; Okada et al., 1999; Lucas et al., 2003; Trebak et al., 2003; Estacion et al., 2006), a property apparently unique to TRPC channels. An alternative model, based on whole-cell recordings, in which light-induced reduction of PIP_2 in combination with acidification would be responsible for TRP gating while DAG would not have a direct effect has also been postulated (Huang et al., 2010). However, this model cannot account for the results reported here and in the previous work of our laboratory. Delgado and collaborators (Delgado et al., 2014) reported that adding PIP_2 to excised patches pre-exposed to illumination, which presented high TRP activity and presumably contained low levels of the phospholipid and high levels of DAG, had no effect on channel activity, contrary to expectation from the latter model.

TRP gating may involve binding of the lipid messenger to the TRP channels, light-dependent changes in mechanical forces on the channel via membrane lipids (Huang et al., 2010; Hardie and Franze, 2012), changes in membrane curvature or fluidity exerting mechanical forces on TRP (Hardie and Franze, 2012; Liu and Montell, 2015), changes in redox environment, and/or conformational changes in other scaffolding-associated proteins. It is also possible that tension forces transmitted through conformational changes in and/or among the transducisomal proteins increase TRP open probability. The excised rhabdomeric membrane patches are a promising system to address this question at the molecular level.

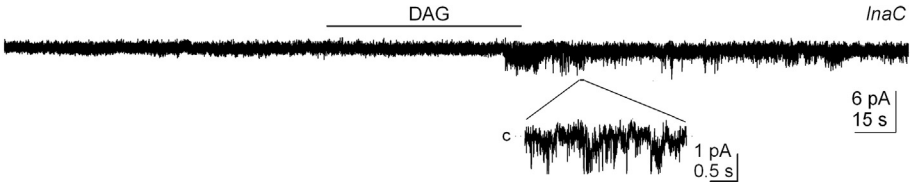

Fig. 4. Protein kinase C is not required for the activation of TRP by DAG. (A, B) Recordings from a patch excised from flies lacking PKC (*inaC*). DAG induced channel activation in spite of the lack of PKC (N = 4). Vp: 40 mV.

We reported previously (Delgado and Bacigalupo, 2009) that the delay to channel opening following light stimulation in on-cell patches was quite long compared with the normal light response (seconds vs. tens of mil-

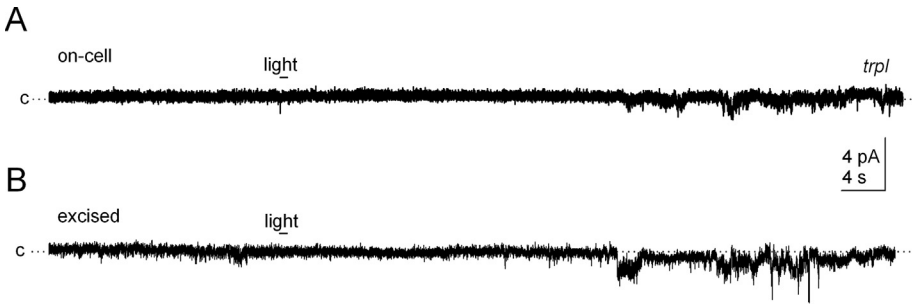

Fig. 5. Light can activate TRP in excised patch. (A) Light-induced activation of TRP in an on-rhabdomeric patch before excising. (B) Similar response of the same patch after excision, in the presence of GTP (1 mM). 23 on-rhabdomeric patches responded to light. 13 of them could be also tested after excising, of which nine responded to light (on-cell: 6.7 ± 1.4 s; excised, 7.2 ± 2.9 s). Vp: 40 mV. Fly was *trpl*$^{-}$. The spontaneous activity after excision, measured as average patch membrane current in pA ± SD over a 5 s duration, was as follows: Before the light response: 0.06 ± 0.16; during the light response: 0.57 ± 0.37; after recovery from light response: 0.00 ± 0.09. The spontaneous activity before excision, was as follows: Before the light response: 0.16 ± 0.07; during the light response: 0.31 ± 0.07; after recovery from light response: 0.16 ± 0.08.

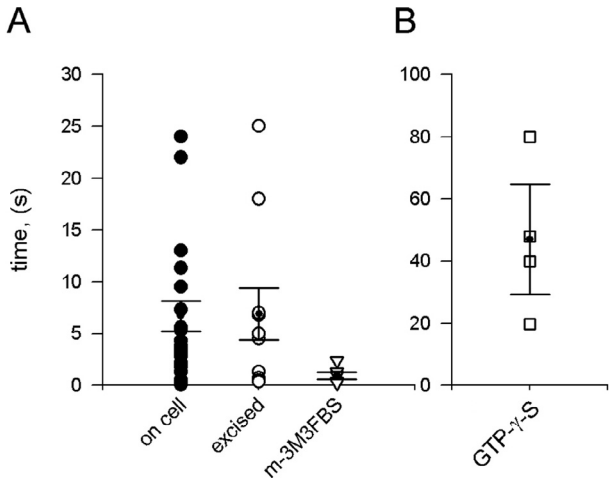

Fig. 6. Quantification of the latencies of all the experimental data collected from patches in each tested condition. The plot on the left comprises the data from the light-activated on-cell patches on the left (black symbols), the light-activated excised patches on the center (white symbols) and the patches activated by m-3M3FBS on the right (triangles). The plot on the right contains the data from the patches activated by GTP-γ-S, in a different scale.

liseconds). Fig. 5 shows that this was also the case for excised patches (on-cell: 6.7 ± 1.4 s; excised: 6.9 ± 2.9 s). We do not know the cause of this long delay. It is possible that transduction components are unevenly distributed over the microvillar membrane, being at much lower density at the microvillar tips where the patch electrode samples channel currents, so that much fewer Rhs or other upstream components would be available to initiate cascades. Alternatively, it is also plausible that sealing of the pipette on the rhabdomeric surface might disrupt the normal and critical structural molecular organization of the phototransduction proteins, affecting the kinetics of their interactions.

Similar long latencies preceded physiological effects of adding DAG and pharmacological agents to our excised patches. Importantly, such long delays are characteristic of lipid-mediated signaling systems studied in reduced preparations. TRP activation by externally applied PUFAs in whole-cell recordings exhibited > 10 s delay (Chyb et al., 1999). Activation of the mechanosensitive channel by lysophosphatidylcholine in excised patches from liposomes showed similar latency (Vasquez et al., 2008). These delays are likely due to slow partitioning of lipidic molecules into the membrane, and the variability in the delays may reflect differences in solubilities. Accordingly, in experiments that circumvent the partitioning issue, Leinders-Zufall and colleagues (Leinders-Zufall et al., 2018) found, in chemosensory neurons pre-incubated with the photo-activatable DAG-analog, PhoDAG-3, that TRPC2 in excised patches opened ~12 ms after photo-activation of DAG. Thus, the issue of the latency remains to be clarified with future work.

It is thought that the response to a single photon occurs in a single microvillus and it seems likely that this unitary response to light arises as a summation of local transduction events within the microvillus (Scott and Zuker, 1998; Hardie and Juusola, 2015). Because of the compact lateral association of the microvilli in the rhabdomere, it is reasonable to assume that the excised patches mostly contain the tips of the microvilli enclosed within the rim of the pipette. We estimate that there are ~100 microvilli tips (~0.04 μm diameter each) within an excised patch and that the area of a microvillus tip is ~1/100 the area of the total microvillus. If one microvillus has 100 INAD, 100 TRP channels, 100 PLC and 4,000 Rhs, as suggested in blowflies (Huber et al., 1996; Bahner et al., 2000), there would be a similar number of these proteins in one patch and a microvillus tip would contain, on average, 1 transducisome and 40 Rhs, assuming a homogeneous distribution of components throughout the organelle. Mysteriously, we consistently observed that, in both on-cell and excised patches stimulated with light, the number of channels opened was generally smaller than that evoked pharmacologically tested. The reason for this observation remains to be investigated.

Our overall results provide strong support for DAG as membrane soluble messenger linking light-induced lipid chemistry with TRP channel opening. The excised rhabdomeral patch system has very important advantages: (i) it sustains a metabotropic cascade in a functional state in its native membrane in isolation; (ii) it is much simpler than whole-cell preparations; (iii) it provides access to the cytoplasmic side of the patch for endogenous as well as pharmacological agents; (iv) it allows direct recordings of TRP and TRPL channels at a molecular level in the native membrane, in the absence of any other channels; (v) the natural stimulus (light) can be applied with precisely determined intensity, duration, and ON and OFF times, in contrast to

metabotropic systems that are triggered by extracellular agonists requiring access to the extracellular membrane surface hidden within the pipette.

In conclusion, our finding that TRP can be activated by light in excised patches confirms the presence and functionality of each of the transduction components required for TRP opening.

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