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Subcellular translocation of the eGFP-tagged TRPL channel in *Drosophila* photoreceptors requires activation of the phototransduction cascade

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Summary

Signal-mediated translocation of transient receptor potential (TRP) channels is a novel mechanism to fine tune a variety of signaling pathways including neuronal path finding and *Drosophila* photoreception. In *Drosophila* phototransduction the cation channels TRP and TRP-like (TRPL) are the targets of a prototypical G protein-coupled signaling pathway. We have recently found that the TRPL channel translocates between the rhabdomere and the cell body in a light-dependent manner. This translocation modifies the ion channel composition of the signaling membrane and induces long-term adaptation. However, the molecular mechanism underlying TRPL translocation remains unclear. Here we report that eGFP-tagged TRPL expressed in the photoreceptor cells formed functional ion channels with properties of the native channels, whereas TRPL-eGFP translocation could be directly visualized in intact eyes. TRPL-eGFP failed to translocate to the cell body in flies carrying severe mutations in essential phototransduction proteins, including rhodopsin, Gαq, phospholipase Cβ and the TRP ion channel, or in proteins required for TRP function. Our data, furthermore, show that the activation of a small fraction of rhodopsin and of residual amounts of the Gq protein is sufficient to trigger TRPL-eGFP internalization. In addition, we found that endocytosis of TRPL-eGFP occurs independently of dynamin, whereas a mutation of the unconventional myosin III, NINAC, hinders complete translocation of TRPL-eGFP to the cell body. Altogether, this study revealed that activation of the phototransduction cascade is mandatory for TRPL internalization, suggesting a critical role for the light induced conductance increase and the ensuing Ca^{2+}-influx in the translocation process. The critical role of Ca^{2+} influx was directly demonstrated when the light-induced TRPL-eGFP translocation was blocked by removing extracellular Ca^{2+}.

Keywords

Drosophila; eGFP; Endocytosis; G Protein; Phototransduction; TRP channel

Introduction

TRP channels constitute a large and diverse protein family of cation channels, which is conserved through evolution (for reviews, see Minke and Cook, 2002;Clapham, 2003;Clapham et al., 2003;Montell, 2003;Moran et al., 2004;Montell, 2005). TRP channels are employed in a number of sensory systems, including invertebrate vision, mechanoreception, taste perception, nociception, thermoreception, pheromone detection and in non-neuronal cells,

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where they function in vasorelaxation or in cell cycle control. *Drosophila* TRP, which is the founder of this ion channel family, and its homologue TRP-like (TRPL) are required for visual transduction. The physiological site of action of TRP and TRPL is the rhabdomeral photoreceptor membrane formed by a densely packed stack of microvilli along the side of the photoreceptor cells in the fly compound eye. The cation channels are activated in response to light absorption by the visual pigment rhodopsin through a Gq protein-mediated signaling pathway (Devary et al., 1987;Bloomquist et al., 1988;Ranganathan et al., 1995;Montell, 1999;Hardie and Raghu, 2001). Although the exact gating mechanism of TRP and TRPL is not yet known, phospholipase Cβ that hydrolyzes phosphatidylinositol 4,5-bisphosphate to form the second messengers diacylglycerol and 1,4,5-inositol trisphosphate is mandatory for the activation of the ion channels. Both second messengers have been implicated in TRP and TRPL activation [see Hardie (Hardie, 2003) and Minke and Agam (Minke and Agam, 2003) for a discussion of possible gating mechanisms].

We reported previously that TRPL undergoes a light-regulated subcellular translocation. High level of rhabdomeral TRPL characteristic of dark-raised flies was reduced to a low level upon continuous illumination, whereas the content of rhabdomeral TRP is not altered by exposing *Drosophila* to different light conditions (Bähner et al., 2002). The change of the TRP/TRPL ratio has physiological consequences. Flies with high TRPL level in the rhabdomere respond to a wider range of light intensities than flies with a reduced TRPL content, and they are more sensitive to adaptation by dim background lights (Bähner et al., 2002). Translocation of the *Drosophila* TRPL channel thus constitutes a powerful in vivo model system for studying the still unclear mechanisms underlying translocation of mammalian TRPC and TRPV channels that have been recently reported (Kanzaki et al., 1999;Bezzerides et al., 2004).

To understand further the cellular mechanism underlying TRPL translocation we generated transgenic *Drosophila,* which express an eGFP-tagged TRPL channel in photoreceptors R1–6. We found that TRPL-eGFP forms functional ion channels with native properties. The analysis of TRPL-eGFP translocation in various mutants with defects in phototransduction proteins shows that activation of rhodopsin and of downstream signaling proteins of the phototransduction cascade is mandatory for inducing TRPL internalization.

Results

eGFP-tagged TRPL is relocated in a light-dependent way

For the expression of an eGFP-tagged TRPL ion channel in photoreceptor cells R1–6 of the compound eye, a DNA construct containing the promoter of *Drosophila* rhodopsin 1 (Rh1) and the fused coding sequences for TRPL and the enhanced green fluorescent protein (eGFP) was cloned into a P-element vector and used to generate transgenic *Drosophila*. Western blot analysis with anti-TRPL antibodies revealed that flies that contain this transgene in a wild-type background express native TRPL (molecular mass 128 kDa) and TRPL-eGFP (Fig. 1A). Two protein bands with an apparent molecular mass of ~145 kDa and 135 kDa, representing TRPL-eGFP, were detected in flies carrying the TRPL-eGFP transgene (Fig. 1A, left panel). The protein bands corresponding to TRPL-eGFP were also recognized by an anti-GFP antibody (Fig. 1A, middle panel). The second, lower molecular mass band is likely to represent a degradation product of the full-length protein. The amount of the second cation channel subunit, TRP, present in the photoreceptor cells is not significantly affected by the expression of TRPL-eGFP (Fig. 1A, right panel).

When flies expressing the TRPL-eGFP reporter gene were kept in the dark for 16 hours and were then inspected at low magnification under a fluorescence microscope, a very bright green fluorescing deep pseudopupil (dpp) was observed (Fig. 1B). The dpp is a virtual image consisting of seven spots that reflect the trapezoidal pattern of the rhabdomeres in a single

ommatidium. It is generated by the superimposed images of several rhabdomeres of photoreceptor cells from different ommatidia which have the same optical axis (Franceschini and Kirschfeld, 1971b). Since the Rh1 promoter drives TRPL-eGFP expression only in R1–6 cells, a central spot corresponding to the rhabdomere of R7 and R8 photoreceptor cells was missing in the fluorescing dpp of the transgenic flies and served as an internal control. The eGFP-fluorescence of the dpp in flies kept in the dark suggests that TRPL-eGFP is located in the rhabdomeres under this condition. A different picture was obtained when the flies were kept in the light. In these flies no fluorescing dpp was observed, suggesting that TRPL-eGFP was not present in the rhabdomeres of the photoreceptor cells (Fig. 1B). These findings are fully compatible with the notion that TRPL-eGFP undergoes a light-dependent translocation between the rhabdomere and an intracellular compartment.

A high resolution image of the cellular TRPL-eGFP localization can be obtained when the cornea of the eye is optically neutralized by water immersion as has been described by Franceschini et al. (Franceschini and Kirschfeld, 1971a;Franceschini et al., 1981). This method allows visualization of single rhabdomeres and cell bodies of single ommatidia in a living fly. We used a water immersion objective to study the distribution of TRPL-eGFP in transgenic flies which were kept in the dark or in the light for 16 hours (Fig. 1C). In flies kept in the dark, eGFP-fluorescence was restricted to the rhabdomeres of photoreceptor cells R1–6, whereas flies kept in the light showed fluorescence in the cell bodies, but not in the rhabdomeres (which appear dark in Fig. 1C right). This light-dependent redistribution of TRPL-eGFP was further confirmed when compared to the redistribution of native TRPL by analyzing eGFP fluorescence and immunofluorescence of antibodies against TRPL in cross sections through *Drosophila* eyes (supplementary material Fig. S1). A similar distribution in the rhabdomeres or in the cell bodies was observed for native TRPL and TRPL-eGFP in dark- or light-raised flies, respectively.

In order to determine the kinetics of TRPL-eGFP relocation, we quantified the intensity of eGFP fluorescence inside and outside the rhabdomere as a function of time, using fluorescent images obtained from intact flies with the water immersion technique. The obtained time courses (Fig. 2A, B) revealed that the internalization of TRPL-eGFP in the light and its movement into the rhabdomeres in the dark occurred within several hours with half-times of 3.25 hours and 1 hour, respectively. The light-dependent redistribution of *Calliphora* TRPL reported previously was significantly shorter (Bähner et al., 2002). This quantitative difference may have resulted from species differences. Qualitative evaluation of the time-dependent translocation of native *Drosophila* TRPL by immunocytochemistry suggests a similar time course for the translocation of native TRPL and TRPL-eGFP in *Drosophila* (supplementary material Fig. S2).

In another stimulation paradigm we subjected either dark-or light-adapted flies to alternating light and dark intervals (30 minutes each). Under these conditions an equilibrium was established in which about 75% of TRPL-eGFP was located in the rhabdomeres whether the flies were light- or dark-adapted before the experiment (Fig. 2C). This is in line with our findings showing that the internalization of TRPL-eGFP is slower than its translocation to the rhabdomeres. Finally, we investigated TRPL-eGFP internalization in different light intensities (supplementary material Fig. S3). Even under the lowest light intensity tested (2 Lux) complete internalization of TRPL-eGFP was observed in most photoreceptor cells. However, under this condition few photoreceptor cells were observed in which TRPL-eGFP was located completely in the rhabdomeres.

TRPL-eGFP forms functional ion channels

In order to determine whether or not the eGFP-tag of TRPL interferes with the formation of functional ion channels, we generated flies that expressed TRPL-eGFP in a trp^{P343}; $trpl^{302}$

double mutant background. The trp^{P343}; $trpl^{302}$ double mutant lacks functional photoreceptor channels and does not respond to light at all (Niemeyer et al., 1996;Scott et al., 1997). Thus, the presence of light responses in flies that express TRPL-eGFP in this genetic background would indicate the formation of functional TRPL-eGFP channels. We examined the properties of TRPL-eGFP channels electrophysiologically at two levels: (i) in vivo, using the electroretinogram (ERG) and (ii) in situ, using patch-clamp whole cell recordings from isolated ommatidia. ERG-recordings from TRPL-eGFP-expressing mutants revealed responses to medium intensity lights, which declined to that of the dark baseline during illumination, within 5 seconds. The response to light recovered in the dark as revealed by a significant transient response to a second light stimulus applied 10 seconds after the first light. (Fig. 3A, middle trace). The transient light-responses from TRPL-eGFP-expressing flies are reminiscent of light-responses obtained from *trp* mutants that express only native TRPL channels (Fig. 3A bottom trace). The light responses of both TRPL-eGFP-expressing flies and *trp* mutants are very different from that of wild-type (WT) flies, which have a sustained response during illumination and the responses to the first and second light pulses are virtually identical (Fig. 3A, upper trace). To further compare the physiological properties of TRPL-eGFP-expressing flies and *trp* mutants, intensity-response relationships (V-log I curves) were plotted from the peak amplitude of ERG recordings in response to increased intensities of orange light. V-log I curves of mutants expressing either native TRPL or TRPL-eGFP were almost indistinguishable (Fig. 3B). In both mutants the V-log I curve was shifted to higher levels of light intensity by about 1 log unit as compared to wild-type flies. A quantitative difference between TRPL- and TRPL-eGFP-expressing flies was found in the amplitude of the response to a second light pulse that was applied 10 seconds after the first pulse (Fig. 3A, C). Responses of TRPL-eGFP-expressing flies to the second light pulse were significantly larger than the corresponding responses of *trp* mutants, indicating that the photoresponse of the latter recovered slower from the light-induced inactivation (Fig. 3A, C). The kinetics of the dark recovery from response inactivation is one of the hallmarks of the *trp* mutant phenotype, where slower recovery corresponds to stronger *trp* phenotype (Minke, 1982). It is possible that the observed difference in the responses to a second light pulse resulted from differences in the amounts of TRPL or TRPL-eGFP present in the corresponding photoreceptor cells.

To compare more directly the characteristics of the native TRPL to TRPL-eGFP channels, light-induced currents (LIC) were measured in TRPL-eGFP-expressing flies and *trp* mutants. The waveforms of the LIC in response to increasing light intensities were very similar in both fly strains (data not shown). Furthermore, both strains revealed virtually identical current-voltage relationship and the reversal potential (which is the membrane voltage in which the light-induced inward current changes to outward current in response to the constant light pulse) was ~0 mV in both fly strains (Fig. 3D) (Hardie and Minke, 1992). Altogether, the physiological properties of the TRPL-eGFP and the native TRPL channels were very similar, indicating that the attachment of the eGFP-tag to the TRPL channel did not modify the functional properties of the TRPL channel both in vivo and in situ.

The light-dependent translocation of TRPL requires functional rhodopsin

In an attempt to determine how the light signal leads to the translocation of the TRPL channel we examined whether rhodopsin is required for this process and whether the efficiency of TRPL relocation is proportional to the amount of activated rhodopsin. Fig. 4 shows that TRPL-eGFP failed to move following illumination with orange light in the *ninaE* mutant, which lacks Rh1 (O'Tousa et al., 1985;Zuker et al., 1985) (Fig. 4C, D). This experiment showed that rhodopsin, a major protein of the microvilli, is required for light-dependent TRPL-eGFP internalization. This result did not rule out the possibility that the defect in TRPL-eGFP internalization is due to a requirement of Rh1 for proper rhabdomere formation rather than its function as a photon capturing protein. To distinguish between these possibilities we made use of a *Drosophila*

mutant that ectopically expresses the UV-sensitive rhodopsin Rh3 in the major photoreceptor cells of a *ninaE* mutant (Feiler et al., 1992). The presence of Rh3 in R1–6 photoreceptor cells rescues the morphological defect of the rhabdomeres, but Rh3 cannot be activated by long wavelength light (Feiler et al., 1992). Illumination with orange light resulted in internalization of TRPL-eGFP in wild-type flies (Fig. 4A, B) but not in flies carrying the UV-sensitive Rh3 instead of the native Rh1 (Fig. 4E, F). However, partial internalization of TRPL-eGFP was observed when the Rh3-expressing flies were illuminated with UV (Fig. 4G). These results show that activation of rhodopsin is required for TRPL internalization and identifies rhodopsin as the light-sensor that initiates the relocation of the ion channel. The mutant $ninaB^{P315}$ cannot form the retinal chromophore from carotenoids and shows a dramatically reduced amount of opsin protein on western blots (von Lintig et al., 2001). In this mutant we observed variable degrees of TRPL-eGFP translocation (Fig. 4H–J). Whereas in some individual flies orange-light illumination resulted in almost complete translocation of TRPL-eGFP to the cell body, in other individuals TRPL-eGFP was confined to the rhabdomeres after illumination. Dark-raised flies always displayed rhabdomeral localization of TRPL-eGFP. A plausible explanation for the above variability is that $ninaB^{P315}$ flies differ in the individual content of rhodopsin generated from carotenoid-independent sources of retinal, and hence show variable TRPL-eGFP translocation phenotypes.

Activated rhodopsin (i.e. in its metarhodopsin state) is removed from the rhabdomeral membrane in a process that requires the formation of Arrestin 2-metarhodopsin complexes and binding to clathrin-coated vesicles (Dolph et al., 1993;Alloway et al., 2000;Kiselev et al., 2000). A question arises as to whether TRPL-eGFP is internalized with activated rhodopsin, in the same endocytotic vesicles. If this were the case, the efficiency of TRPL internalization should be proportional to the amount of activated rhodopsin generated by illumination. The difference in the absorption spectra of rhodopsin and metarhodopsin lead to production of a very small amount (<2%) of metarhodopsin during orange illumination, whereas green and blue light convert about 50% or 70% of rhodopsin into metarhodopsin, respectively. We, therefore, exposed TRPL-eGFP-expressing flies to orange, green and blue light for 16 hours, and found, by evaluation of TRPL-eGFP-localization with the water immersion method, that each light condition resulted in significant TRPL-eGFP internalization. However, orange light was the most efficient in removing TRPL-eGFP from the rhabdomere, thus indicating that there is no correlation between TRPL translocation and internalization of the photopigment (Fig. 4K). For this reason orange light was used for inducing TRPL internalization in all the experiments described in this paper, except when noted otherwise. Taken together, the results indicate that rhodopsin is necessary for triggering TRPL internalization, which is completely inhibited in the rhodopsin null mutant $ninaE^{17}$. Furthermore, the data suggest that continuous activation of a relatively small percentage of wild-type rhodopsin by orange light or activation of residual amounts of rhodopsin present in some individual flies of the $ninaB^{P315}$ mutant are sufficient for triggering TRPL internalization.

Role of arrestins

Two arrestins, Arrestin 1 (Arr1) and Arrestin 2 (Arr2), are expressed in *Drosophila* photoreceptor cells. According to a recent report Arr1 is required for light-dependent endocytosis of rhodopsin (Satoh and Ready, 2005), whereas the major function of Arr2 is inactivation of activated rhodopsin (Dolph et al., 1993). In addition to visual G protein-coupled receptors, arrestins have also been implicated in the internalization of Frizzled and TGF-β receptors (Chen et al., 2003a;Chen et al., 2003b). Investigation of TRPL-eGFP translocation in *arr1* null mutant revealed no significant difference from wild-type photoreceptors (Fig. 5A, B, G) and only partial impairment of TRPL-eGFP translocation was observed in the *arr2* null mutant (Fig. 5C, D, G). However, translocation of TRPL-eGFP was severely inhibited in the *arr1; arr2* double mutant (Fig. 5E–G), in which we observed TRPL-eGFP in the rhabdomeres

of both light- and dark-raised flies. Hence, the presence of either Arr1 or Arr2 is sufficient at least for partial TRPL-eGFP internalization whereas the lack of both arrestins interferes with proper translocation of TRPL-eGFP.

Mutations in essential phototransduction proteins inhibit TRPL-eGFP translocation

Each one of the three signaling proteins, Gq, PLC and INAD is essential for the generation of the light-induced current and these three proteins together with TRP are essential for the ensuing Ca^{2+} influx. It is therefore important to examine whether these proteins are required for TRPL translocation.

In order to test a possible requirement of the visual G protein for TRPL translocation we used a hypomorphic mutant in the Gα subunit, $Gαq^1$, which produces ~1% of the wild-type levels and shows a dramatic (~1000-fold) loss in light sensitivity (Scott et al., 1995). In most of the photoreceptor cells (typically five out of the six R1–6 cells) TRPL-eGFP was translocated to the cell body as in wild type when the flies were raised in the light (Fig. 6A, B). On average, ommatidia contained 0.95±0.01 photoreceptor cells in which TRPL-eGFP remained completely in the rhabdomere. The amount of Gαq present in individual photoreceptor cells of the $Gαq^1$ mutant may vary and may fall below a value required for TRPL-eGFP internalization in some cells, whereas most cells still contain enough G protein to efficiently trigger TRPL internalization. A similar all-or-none response as in the Gαq mutant was observed when internalization of TRPL-eGFP was triggered with very dim orange light (2 Lux) illumination (see supplementary material Fig. S2). In order to further examine this possibility we generated flies carrying the mutated Gαq allele over a deletion ($Gαq^1$/Df(2R)vg135). This mutant displays a significantly larger reduction in light sensitivity (Scott et al., 1995) than the homozygous $Gαq^1$ mutant, suggesting that the photoreceptors of this fly express negligible amounts of Gαq. In $Gαq^1$/Df(2R)vg135 TRPL-eGFP internalization was strongly inhibited. On average, in 3.65±0.43 photoreceptor cells per ommatidium TRPL-eGFP remained in the rhabdomeres, indicating that further reduction of the Gαq content increases the number of cells that fail to show translocation of TRPL-eGFP (Fig. 6C, D). Altogether, Fig. 6A–D strongly suggest that even a dramatic reduction of the level of the G protein to ~1% did not block TRPL-eGFP translocation and, most likely, almost complete elimination of Gαq was required to block TRPL translocation.

Gαq-GTP activates the *norpA*-encoded phospholipase Cβ. In $norpA^{P24}$, an almost complete null mutant of phospholipase Cβ, no light-dependent translocation of the eGFP-tagged TRPL to the cell body was observed (Fig. 6E, F). In another allele of *norpA* ($norpA^{P57}$), which produces ~20% of the amount of PLC of wild-type photoreceptors and is characterized by a photoresponse of normal amplitude but with slow response termination (Pearn et al., 1996;Cook et al., 2000), translocation of TRPL-eGFP from the rhabdomere to the cell body was observed upon illumination (Fig. 6G, H) indicating that a large reduction of PLC is required to block TRPL translocation.

Absence of the major ion channel, TRP, in the trp^{P343} mutant resulted in the complete loss of the translocation of TRPL-eGFP from the rhabdomere to the cell body (Fig. 6I, J). Inhibition of TRPL-eGFP internalization was also observed in the null mutant of the INAD scaffold protein $inaD^1$. INAD is essential for maintained localization of PLC and TRP to the rhabdomere (Chevesich et al., 1997;Tsunoda et al., 1997), (Fig. 6K, L). Accordingly, the absence of INAD affects two proteins that are essential for TRPL movement and thus its absence was expected to block TRPL translocation.

The *inaF* mutant shows a severe reduction in the amount of TRP and the function of the remaining TRP molecules is impaired (Li et al., 1999). Fig. 6M, N shows that TRPL translocation is blocked in this mutant. It thus confirmed that TRP function is required for

TRPL translocation. Additional support for this conclusion came from studies of a mutant with impaired light control of TRP gating. The mutant trp^{P365} expresses constitutively active TRP channels (Yoon et al., 2000). Photoreceptors of trp^{P365} show fast and severe degeneration in flies homozygous for this allele, owing to uncontrolled Ca^{2+}-influx into the cell (Yoon et al., 2000;Wang et al., 2005). Heterozygous mutants kept in the dark maintain intact photoreceptors for up to 4 days post-eclosion. We investigated the localization of TRPL-eGFP in a trp^{P365}/+ background and found that it localized outside the rhabdomeres in light- and dark-raised flies (Fig. 6O, P). This result strongly suggests that light-independent activation of TRP can lead to localization of TRPL-eGFP in the cell body.

Previously, a requirement for activation of phospholipase Cβ and of the visual G protein for TRPL translocation was ruled out because it was shown that native TRPL is translocated in $norpA^{P24}$ (Bähner et al., 2002) and in $G\alpha q^{1}$ (Cronin et al., 2004) mutants. In this study we show that a reduction of Gαq to 1% or of PLCβ to 20% of the wild-type level did not inhibit TRPL-eGFP translocation in most photoreceptor cells. Much larger reduction in the levels of these signaling proteins in $G\alpha q^{1}/Df(2R)vg^{135}$ and $norpA^{P24}$, respectively, was required to interfere with TRPL-eGFP translocation. The complete inhibition of light-induced TRPL-eGFP translocation by the $norpA^{P24}$ mutation (Fig. 6E, F) and the reported translocation of the native TRPL in the same mutant (Bähner et al., 2002) can be explained by a reduced sensitivity to activation of phototransduction of the TRPL-eGFP internalization process as compared to that of the native TRPL. The $norpA^{P24}$ mutant and also the $inaD^{1}$ mutant show very small but significant activation of the phototransduction cascade (Hardie et al., 2003;Tsunoda et al., 1997), which seems to be sufficient to trigger translocation of native TRPL. Alternatively, the discrepancy between native and eGFP-tagged TRPL might imply the existence of a distinct *norpA*-independent mechanism that is specifically compromised for TRPL-eGFP.

Removal of external Ca^{2+} blocks light-induced TRPL-eGFP translocation

To examine directly whether light-induced Ca^{2+} influx is necessary for TRPL-eGFP translocation, we compared light-induced TRPL-eGFP translocation in the presence and absence of Ca^{2+} in the extracellular medium. To this end we incubated sliced heads of $trpl^{302}$; *trpl-eGFP* flies in oxygenated solutions containing either 1 mM Ca^{2+} or 1 mM EGTA (no Ca^{2+} added). The incubated heads were illuminated with orange light (OG 590 edge filter) for 4 hours. After incubation and illumination, the retinas were isolated and examined under a confocal microscope. Fig. 7B, E shows a very significant translocation of TRPL-eGFP to the base of the rhabdomeres that was observed in illuminated retinas incubated with extracellular Ca^{2+}. Removal of extracellular Ca^{2+} by EGTA completely blocked the light-induced translocation of TRPL-eGFP (Fig. 7A, D), and the localization of TRPL-eGFP remained very similar to its localization in retinas of dark-raised flies (Fig. 7C, D). Fig. 7 thus demonstrates that removal of extracellular Ca^{2+} inhibits light-induced translocation of TRPL-eGFP.

Internalization of TRPL-eGFP is independent of dynamin

Towards gaining some insight into the cell biological mechanism which may underlie TRPL internalization we studied the dependence of TRPL-translocation in the mutant *shibire* (*shi*). *shi* encodes a dynamin required for budding-off of vesicles from the membrane. A temperature-sensitive mutant (shi^{ts1}) fails to recycle synaptic vesicles at the restrictive temperature and renders the flies paralyzed. TRPL-eGFP translocation was unaffected in shi^{ts1} indicating that the internalization mechanism of TRPL-eGFP is dynamin-independent (Fig. 8A, B). In $ninaC^{5}$ that is a null-mutant of an unconventional myosin III, TRPL-eGFP was properly localized in the rhabdomeres in dark-raised flies. Illumination of the $ninaC^{5}$ mutant with orange light resulted in only partial internalization of the ion channel (Fig. 8C, D). Thus, this protein, which affects organization of the cytoskeleton (Matsumoto et al., 1987) is not required for the

incorporation of TRPL-eGFP into the rhabdomere, but its mutation affects translocation of TRPL-eGFP to the cell body.

Discussion

The light-dependent translocation of TRPL in *Drosophila* photoreceptor cells alters the composition of ion channels in the rhabdomeral membrane. Thus, the rhabdomeral membrane contains functional TRP and TRPL channels in dark-raised flies but harbors only functional TRP in light-raised flies because of the reduced number of TRPL channels (Bähner et al., 2002) and because the Ca^{2+}-influx via the TRP channels inactivates the remaining TRPL channels (Reuss et al., 1997).

Identification of the triggering signal, which initiates the specific internalization of TRPL is important for understanding TRPL translocation. One possible triggering mechanism is the light-dependent activation of the TRPL channels themselves, which could form an ion channel conformation susceptible for internalization. Other possibilities include activation of TRPL internalization by increase in intracellular Ca^{2+} concentration, which results from Ca^{2+} influx through activated TRP, or internalization of TRPL together with metarhodopsin in an arrestin-dependent way. Our findings that functional rhodopsin is required for TRPL-eGFP internalization are in agreement with each one of these three possibilities. However, since activation of a small fraction of the rhodopsin molecules by orange light in wild-type flies was sufficient for maximal TRPL-eGFP internalization and since TRPL-eGFP internalization could be triggered by the residual rhodopsin present in the *ninaB*^{P315} mutant, it seems unlikely that removal of TRPL-eGFP from the rhabdomeral membrane is achieved by co-internalization with metarhodopsin.

A recent paper by Satoh and Ready (Satoh and Ready, 2005) reveals that Arr1 and Arr2 have separate functions. Arr1 is required for rhodopsin internalization whereas Arr2 is required for rhodopsin inactivation. Thus, if TRPL-eGFP was internalized together with rhodopsin one would expect that mutation of *arr1* inhibits internalization of TRPL-eGFP. This is not the case. On the other hand, the defect of rhodopsin inactivation in the *arr2* mutant is amplified in the *arr1/arr2* double mutant (Dolph et al., 1993) as is the case with the inhibition of TRPL-eGFP internalization. This correlation may suggest that proper inactivation of rhodopsin is required for complete internalization of TRPL-eGFP. In addition the *arr2* mutant shows light-dependent degeneration of the photoreceptor cells. Although in our experiments we used young flies which did not show any sign of degeneration we can not exclude that this may affect the internalization of TRPL-eGFP.

Our data indicate that activation of signaling proteins such as Gαq, PLCβ and TRP, which operate downstream of rhodopsin in the phototransduction cascade, are essential for TRPL-eGFP translocation. However, activation of a relatively small percentage of these signaling molecules during many minutes triggers complete TRPL-eGFP translocation. Because of the huge amounts of signaling proteins in the photoreceptor cell (Hardie and Right, 2001) and the high gain of phototransduction, activation of a relatively small fraction of the signaling molecules is sufficient to induce nearly saturated responses via opening of the TRP channels. Activation of TRPL alone is not sufficient and possibly not even required for its translocation to the cell body, rather the second ion channel, TRP, must be activated. This is evident from the lack of TRPL-eGFP translocation in the *trp* null mutant and in mutants with altered TRP function, such as *inaD*^{1} and *inaF*^{P106x}. The observed localization of TRPL-eGFP to the cell body irrespective of the light condition in a mutant expressing constitutively active TRP (*trp*^{P365}) suggests that activity of TRP, independent of activation of the phototransduction cascade and independent of the activation of the TRPL channel, causes translocation of TRPL-eGFP to the cell body. Taken together, an increase of the intracellular concentration of Ca^{2+}

through TRP channels in the light is an attractive mechanism for triggering TRPL translocation. This hypothesis is strongly supported by our finding that large reduction of extracellular Ca^{2+} in the eye inhibits light induced TRPL-eGFP internalization.

To further elucidate the mechanism by which TRPL-eGFP is transported from the rhabdomeral membrane to the cell body, we analyzed the possible requirement of dynamin and of the myosin III NINAC for TRPL-eGFP translocation. Dynamin is crucial for the budding of vesicles from the plasma membrane in several endocytotic pathways (Conner and Schmid, 2003). However, vesicular internalization pathways may also be independent of dynamin. For example, for the endocytosis of G protein-coupled receptors dynamin-dependent and dynamin-independent pathways have been reported (Zhang et al., 1996;Pals-Rylaarsdam et al., 1997;Vickery and von Zastrow, 1999). The internalization of TRPL-eGFP is not affected in a temperature-sensitive *shibire* mutant at the restrictive temperature, indicating that the translocation of TRPL-eGFP is dynamin independent. Furthermore, we observed no defect in the translocation of TRPL-eGFP from the cell body to the rhabdomere in a mutant lacking the myosin III NINAC, but TRPL-eGFP translocation in the opposite direction was compromised in this mutant. Because myosin III is a plus ended myosin and the plus ends of actin filaments are oriented towards the tips of the microvilli (Lee and Montell, 2004), it is not feasible that this myosin mediates the transport of proteins out of the rhabdomere. However, the *ninaC*^{5} mutant exhibits secondary defects such as disruption of the actin cytoskeleton and retinal degeneration (Matsumoto et al., 1987;Hicks and Williams, 1992). We suggest that the observed partial inhibition of TRPL-eGFP transport from the rhabdomere to the cell body is due to these secondary defects.

Besides TRPL, at least two other proteins mediating *Drosophila* phototransduction, Arrestin 2 and the visual Gαq, undergo light-dependent translocation between the rhabdomere and the cell body (Byk et al., 1993;Kiselev et al., 2000;Kosloff et al., 2003;Lee et al., 2003;Lee and Montell, 2004). Likewise, in vertebrate photoreceptors arrestin and the visual G protein transducin translocate between the inner and outer segment in a light-dependent way (Arshavsky, 2003). In both visual systems, arrestin and G protein movements occur in opposite directions, that is, in the light arrestin accumulates whereas the G protein is depleted in the photoreceptive membrane and vice versa in the dark. Accordingly, these light-dependent relocations of visual signaling proteins make the photoreceptor more sensitive in the dark and less sensitive in the light and mediate long-term adaptation of the *Drosophila* and vertebrate visual systems (Sokolov et al., 2002;Lee et al., 2003). A third protein that translocates in vertebrate photoreceptors is the Ca^{2+} binding protein recoverin (Strissel et al., 2005).

The mechanisms underlying these protein translocations have been elucidated in part for *Drosophila* arrestin (Lee and Montell, 2004), the Gαq subunit (Kosloff et al., 2003;Cronin et al., 2004;Elia et al., 2005) and for vertebrate transducin (Sokolov et al., 2004). Translocation of *Drosophila* arrestin from the cell body to the rhabdomere has been reported to require the *ninaC*-encoded myosin III which may actively transport arrestin along the actin cytoskeleton of the photoreceptor microvilli through PIP_3-enriched vesicles, to which arrestin binds (Lee and Montell, 2004). However, the requirement of the myosin III NINAC for arrestin translocation has been challenged in a more recent publication (Satoh and Ready, 2005). Removal of arrestin from the rhabdomeral membranes in the dark does not require cytoskeletal elements and may thus occur passively (Lee and Montell, 2004). Likewise, Gαq translocation into the rhabdomere, but not its removal, is facilitated by the myosin III NINAC (Cronin et al., 2004). Translocation of vertebrate transducin is aided by phosducin, an abundant photoreceptor-specific protein that binds to the βγ subunits of transducin (Sokolov et al., 2004). Phosducin increases the solubility of the G protein subunits and may thereby facilitate transducin translocation.

These mechanisms are markedly different from the mechanism underlying TRPL translocation because TRPL is a transmembrane protein that cannot enter the soluble fraction and needs to be removed from the rhabdomere by an endocytotic pathway, whereas arrestin and the visual G protein change from a membrane attached state to a soluble state. Therefore, elucidating the triggering mechanism of TRPL translocation reported in the present study is the first step for unraveling the mechanism underlying an important cellular process.

Materials and Methods

Fly stocks

The following strains and mutants of *Drosophila melanogaster* were used: w Oregon R, *yw; trpl*^{302} (Niemeyer et al., 1996), *yw; trpl*^{302}*; trp*^{P343} (Yang et al., 1998), *yw;;trp*^{P343} (Pak, 1979), *w;;trp*^{P365} (Yoon et al., 2000), *yw; ninaE*^{17} (O'Tousa et al., 1985), *yw; ninaE*^{17}*/P[Rh1 +3]* (Feiler et al., 1992), *w;;ninaB*^{P315} (von Lintig et al., 2001), *arr1*^{1}*cn bw* (Dolph et al., 1993), *w;; arr2*^{3} *st* (Dolph et al., 1993), *w; arr1*^{1} *cn bw; arr2*^{3} (Dolph et al., 1993), *w; Gαq*^{1} (Scott et al., 1995), *Df(2R)vg135, nompA*^{vg135}*/CyO, S* bw*^{1} (Lasko and Pardue, 1988), *w norpA*^{P24} (Bloomquist et al., 1988), *norpA*^{P57}*; bw; st* (Pearn et al., 1996), *yw; inaD*^{1} *cn bw* (Tsunoda et al., 1997), *w inaF*^{P106x} (Li et al., 1999), *w shi* ^{ts1} (Grigliatti et al., 1973), and *w; ninaC*^{5} (Pak, 1979). The transgenic flies expressing TRPL-eGFP, which were generated in this study, were crossed with the mutants to obtain the genotypes indicated in the figure legends. If the mutations were located on the second or third chromosome, the flies were made homozygous for both the mutation and the *trpl-eGFP* transgene, inserted into the other autosome. If the mutation mapped to the X-chromosome, male flies of the F1 generation that were hemizygous for the corresponding mutation and heterozygous for the *trpl-eGFP* transgene were used for analysis.

Flies were raised at 24°C in a 12 hours light/12 hours dark cycle. 12–18 hours before the experiment, flies were kept in the dark, or were illuminated with white light (18 W fluorescent lamp, ~700 Lux), orange light (acrylic glass cut-off filter transmitting light >560 nm, 450 Lux), green light (acrylic glass wide-band filter transmitting light between 460 nm and 610 nm, 30 Lux), blue light (acrylic glass wide-band filter transmitting light between 310 nm and 490 nm, 3 Lux) or with UV (344 nm monochromatic light, xenon high-pressure lamp 150 W). Dark-raised flies were dissected under dim red light (Schott RG 630, cold light source KL1500, Schott, Germany), whereas light-raised flies were dissected under white light.

Generation of DNA constructs and transgenic *Drosophila*

To generate the DNA construct used to express a TRPL-eGFP fusion protein, the stop codon and the 3′ untranslated region of a *trpl* cDNA clone (Phillips et al., 1992) in p BluescriptII SK (Stratagene, Germany) were removed by substituting the sequence 3′ of a *Afl*II restriction site with a PCR fragment containing *Afl*II and *Apa*I cloning sites. The modified *trpl* cDNA was subcloned after partial digestion with *Eco*RI and *Apa*I into a p-Bluescript vector containing a *Drosophila Rh1* promoter fragment (base pairs −833 to +67) (Mismer and Rubin, 1987) and the coding sequence for eGFP (obtained from the vector pEGFP-1, BD Biosciences, Germany). In the resulting construct the *trpl* gene was placed in between the *Rh1* promoter and the eGFP gene to produce a fusion protein in which the eGFP tag was located at the C-terminus of TRPL. *Rh1* promoter, *trpl* and eGFP coding sequences were then cloned into the *Xho*I restriction site of the P-element transformation vector YC4 [a gift from S. Britt, University of Colorado; the YC4 vector is derived from the vector Y.E.S. (Patton et al., 1992)]. P-element-mediated transformation of *Drosophila* was carried out as described previously (O'Tousa, 1992). Host strains used were *Drosophila yellow white* (*yw*) and the *trpl*^{302} mutant in a *yw* background. The transformants were made homozygous for the P-element inserts.

SDS-PAGE and western blot

Western blot analysis was carried out with proteins obtained from homogenates of *Drosophila* heads. Proteins were extracted with 1× SDS-PAGE extraction buffer (4% SDS in 65 mM Tris-HCl, pH 6.8) for 15 minutes at room temperature. Proteins from three heads were subjected to SDS-PAGE according to Laemmli (Laemmli, 1970), using 7.5% polyacrylamide gels (Midget System, Amersham Pharmacia Biotech, Germany). For immunoblotting, proteins were electrophoretically transferred to PVDF membranes (BioRad Laboratories, Germany) and processed as described previously (Huber et al., 2000), except that the ECL Western Blotting Analysis system (GE Healthcare, Germany) was used for signal detection instead of ^{125}I-labeled secondary antibodies. The antibodies used for western blot analysis were α-DmTRP (Bähner et al., 2000), α-DmTRPL (Bähner et al., 2002) and α-GFP (Roche, Germany).

Electrophysiology

Electroretinograms (ERGs) were performed as described previously (Peretz et al., 1994a). ERG traces were recorded using Axon analog to digital converter operated by pClamp 8 software on a PC computer.

To measure light-induced currents, orange light (OG 590 Schott edge filter) from a Xenon high-pressure lamp (75 W) was delivered to isolated ommatidia via the objective lens (40×, Zeiss, Germany) and attenuated up to seven orders of magnitude by neutral density filters. The maximal luminous intensity of the orange light at the level of the ommatidia was about 3.0 log units above the intensity required for a half-maximal response of the R1–6 photoreceptors.

Dissociated ommatidia were prepared from newly eclosed adult flies (<1 hour post-eclosion). Whole-cell patch-clamp recordings were performed as described previously (Hardie and Minke, 1992;Peretz et al., 1994b). Recordings were made at 21°C using patch pipettes of 5–10 MΩ pulled from fiber-filled borosilicate glass capillaries. Series resistance of 7–14 MΩ was carefully compensated (>80%) during all experiments. Signals were amplified with an Axopatch-1D (Axon Instruments) patch-clamp amplifier, sampled at 2 kHz, and filtered below 1 kHz. The bath solution contained (in mM): 120 NaCl, 5 KCl, 10 TES buffer (*N*-Tris-(hydroxymethyl)-methyl-2-amino-ethanesulphonic acid, pH 7.15), 4 $MgSO_4$, 1.5 $CaCl_2$. For all the experiments an internal solution that blocked K^+ channels was used. The whole-cell recording pipette contained (in mM): 120 CsCl, 15 tetraethylammonium (TEA) chloride, 2 $MgSO_4$, 10 TES buffer (pH 7.15), 4 MgATP, 0.4 Na_2GTP, 1 NAD. The external solution was perfused via a perfusion system at a rate of 25 chambers/minute.

Fluorescence microscopy of intact flies and isolated live retina

Flies at the age of 2–4 days expressing TRPL-eGFP were anaesthetized with diethylether for 2 minutes and kept on ice. The flies were then spiked on an insect needle and mounted with plasticine on an object slide. Fluorescence microscopy (microscope: DM LFS, Leica, Germany; light source: ebq 100 dc-1 [100 W], Jena GmbH, Germany) was carried out with a water immersion objective (HCX APO, L 20×/0.5 W U-V-I, Leica, Germany) using the Leica I3 filter set (illumination path: BP 450–490 nm, dichroic mirror/reflector: 510 nm, observation path: LP 515 nm) for excitation and detection of eGFP-fluorescence. The fluorescence was documented with a digital camera (DC200, Leica, Germany).

For observing the fluorescence in the deep pseudopupil the flies were anaesthetized with CO_2 and were then examined with a 5× objective (HC PL Fluotar, 5×/0.15, Leica, Germany) using the same filter set as described above or with a Zeiss Stemi SV 11, equipped with an epifluorescence device.

For quantitative analyses of the amount of TRPL-eGFP located in the rhabdomeres, fluorescence images obtained with the water immersion technique were analyzed with ImageJ 1.32j software (National Institute of Health, USA). The relative amount of TRPL-eGFP present in the rhabdomeres (R) was calculated using the formula $R=(Ir–Ib)/[(Ir–Ib)+(Ic–Ib)]$, where *I*r, *I*b, and *I*c are the fluorescence intensities in the rhabdomeres, in the background, and in the cell body, respectively. The data were normalized to the values obtained for dark raised flies that were set to 100%. Background intensities were determined in the center of the ommatidium where the rhabdomere of the R7/R8 cells is located.

The number of photoreceptor cells of the *Gαq* mutant, in which TRPL-eGFP translocation was inhibited, was determined by counting fluorescing rhabdomeres of 16 to 31 ommatidia per individual fly from three to five light-raised flies.

For the Ca^{2+}-dependent TRPL-eGFP translocation, sliced heads of TRPL-eGFP-expressing flies were placed in oxygenated modified extracellular solution used for electrophysiology and illuminated for 4 hours with orange light. The live retinas were then isolated and examined with LSM (Olympus Fluoview 200 confocal microscope with 60×/0.9 w LUMPlan objective).

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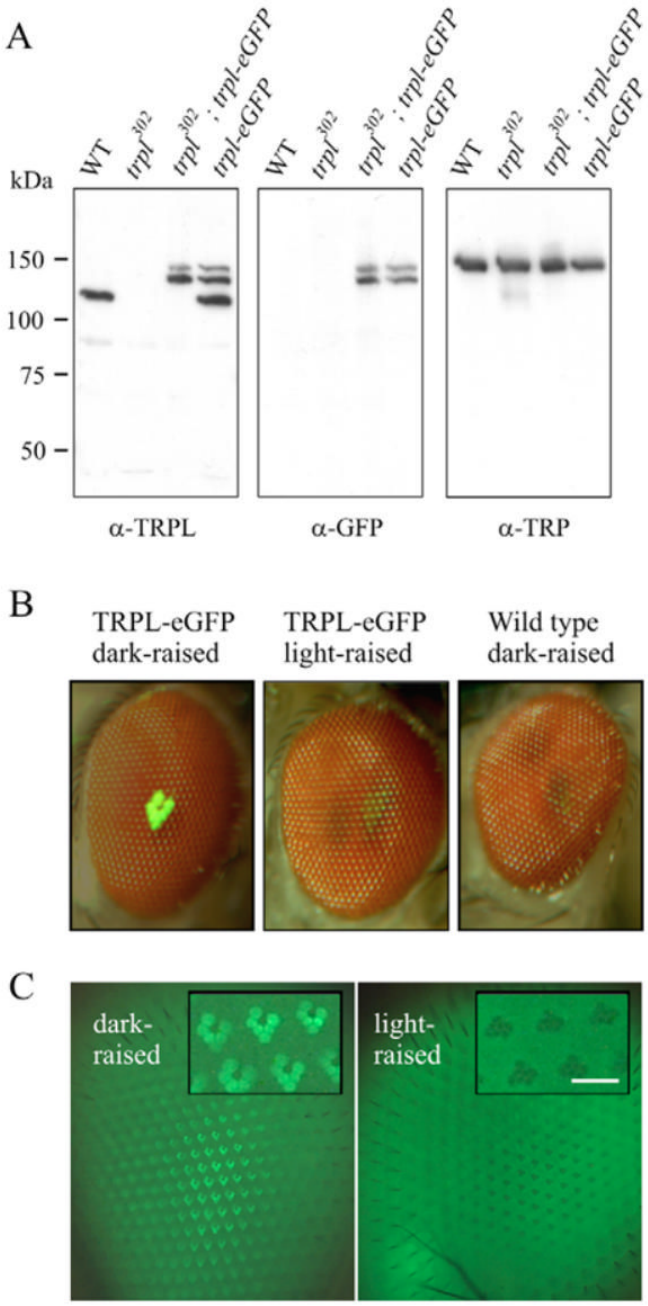

Fig 1.

Expression of TRPL-eGFP in photoreceptors of transgenic *Drosophila*. (A) Western blot analysis of protein extracts obtained from heads of wild-type flies (wt), TRPL-null mutant (*yw; trpl*^{302}), and TRPL-eGFP-expressing flies in TRPL-null (*yw; trpl*^{302}*; trpl-eGFP*) or wild-type background (*yw; trpl-eGFP*). The same blot was probed with antibodies directed against TRPL, TRP and GFP as indicated. Proteins obtained from three *Drosophila* heads were loaded per lane. (B) The green fluorescing deep pseudopupil of TRPL-eGFP-expressing flies (*trpl-eGFP*) raised in the dark (left panel). Fluorescence of the pseudopupil was not observed when the flies were raised in the light (middle panel), nor was it observed in wild-type eyes (right panel). (C) Subcellular localization of TRPL-eGFP in dark- and light-raised transgenic flies

(*yw; trpl-eGFP*). eGFP-fluorescence was detected in intact eyes after optical neutralization of the cornea by water immersion. Flies were kept in the dark (left panel) or under orange light (right panel) for 16 hours. The insets show the central area of the eye at higher magnification. Scale bar, 15 m.

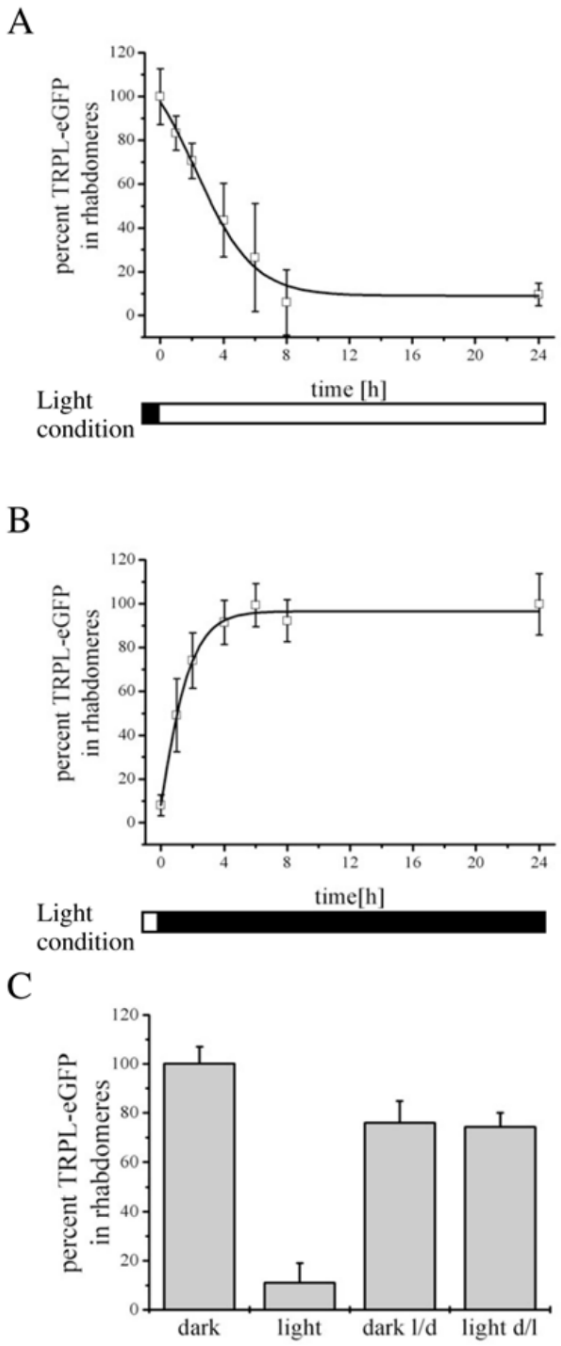

Fig 2.

Time course of TRPL-eGFP translocation. At the indicated time points fluorescence images of intact eyes of flies expressing TRPL-eGFP (*yw; trpl-eGFP*) were obtained using the water immersion technique. From these images the percentage of TRPL-eGFP present in the rhabdomeres at different times after switching the flies from darkness to light (A) or vice versa (B) was calculated (see Materials and Methods). Each data point represents the mean value ± s.d. of at least five independent measurements. (C) Dark-(dark l/d) or light-adapted flies (light d/l) expressing TRPL-eGFP (*yw; trpl-eGFP*) were subjected to alternating light and dark intervals of 30 minutes each for 16 hours. The percentage of TRPL-eGFP present in the

rhabdomeres was determined. The values of dark- and light-adapted flies are shown additionally. Mean values ± s.d. of at least five experiments are shown.

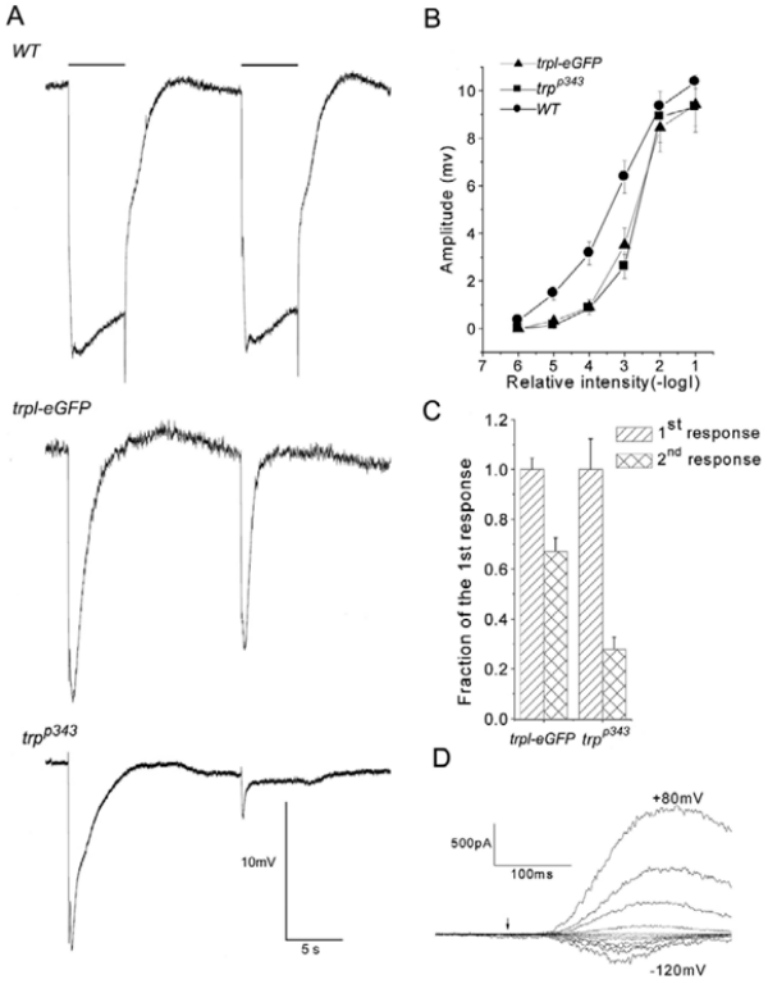

Fig 3.

Fusion of the TRPL channel to eGFP did not affect the physiological properties of the channel in vivo and in situ. (A, upper) ERG recordings from wild-type (WT) flies in response to a pair of orange light stimuli (Schott OG 590 edge filter) with maximal intensity attenuated by 1.0 log unit. (A, middle) ERG recordings from transgenic *Drosophila* expressing TRPL-eGFP fusion protein on null *trpl; trp* background (*yw trpl-eGFP; trpl*^{302}*; trp*^{P343}) in response to the same pair of orange light stimuli used for trace A (upper). There is a transient receptor potential, which declines to baseline within 5 seconds. This light response is typical for *trp* mutants and it is expected from light activation of TRPL channels without the presence of TRP channels. An unusual large response to the second light stimulus after a 10-second dark interval can be observed. (A, bottom) ERG recordings from the null *trp* mutant (*yw;; trp*^{P343}) in a paradigm identical to that of the traces in A (upper and middle). (B) Intensity-response relationship (V-log I curve) measured from WT and the two mutants of Fig. 2A. The peak ERG amplitude was measured as a function of the orange light intensities. The error bars indicate ± s.d. (*n*=9). (C) The peak amplitude of the response to the second stimulus (see traces A middle and bottom) was divided by the peak amplitude of the response to the first stimulus and the averaged ratio calculated from six flies per mutant is presented for the *trpl*-eGFP and *trp*^{P343} mutants. One minute of dark adaptation was used between the pairs of stimuli. (D) The light-induced currents at different membrane potentials are similar in both the TRPL-eGFP-expressing fly and the null *trp* mutant. Whole-cell patch-clamp recordings from isolated ommatidium of *yw trpl-eGFP; trpl*^{302}*; trp*^{P343} fly. Voltage clamp responses to identical orange light pulse of 100

milliseconds duration (Schott OG 590 edge filter with maximal intensity attenuated by 1.0 log unit) delivered at the time indicated by the arrow. The photoreceptor was voltage clamped at membrane potentials between −120 mV and +80 mV in steps of 20 mV. A reversal potential of −1 mV was determined by interpolation after plotting the peak amplitude of the light-induced current as a function of the membrane potential. Very similar reversal potential and similar strongly outward rectifying current voltage relationship was reported for the *trp* mutant (Hardie and Minke, 1992).

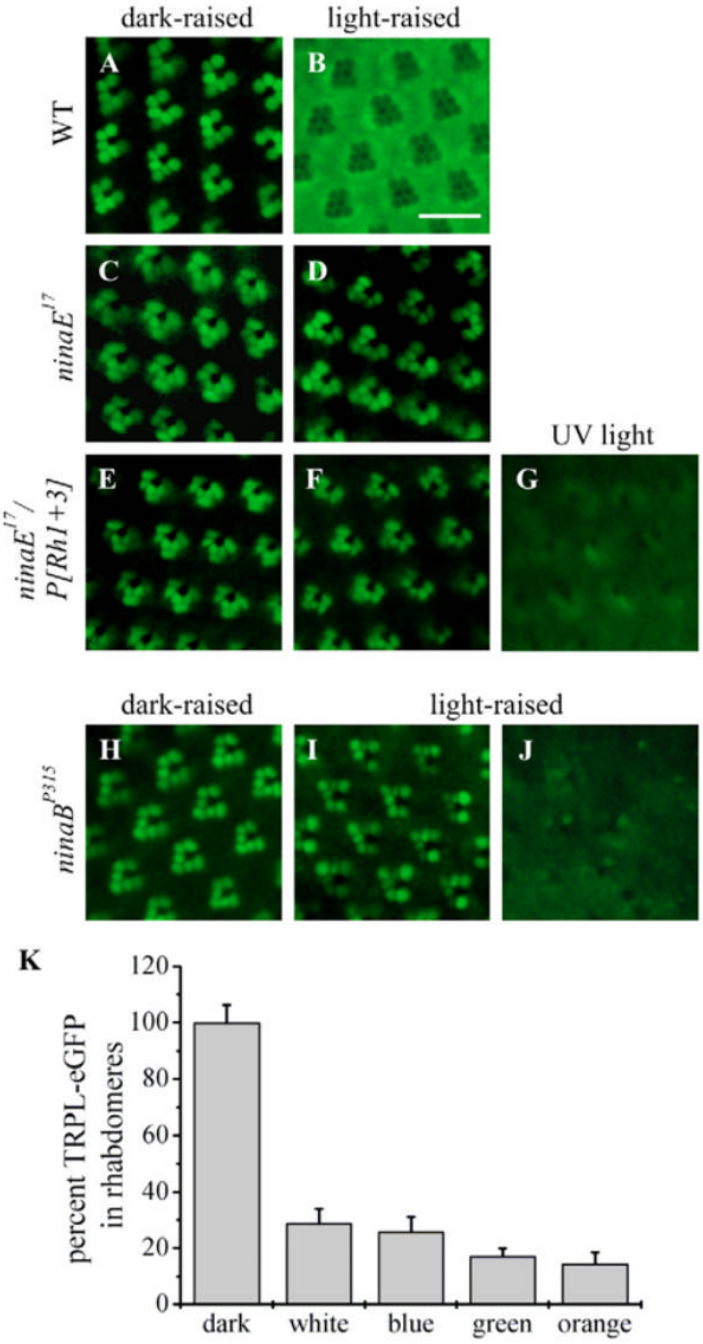

Fig 4.

Functional rhodopsin is required for the light-dependent translocation of TRPL-eGFP. Wild-type (A, B), *yw; ninaE*^{17}*; trpl-eGFP* (C, D), *yw/w; ninaE*^{17}*/P[Rh1+3]; trpl-eGFP* (E, F) and *w; trpl-eGFP; ninaB*^{P315} (H–J) flies expressing TRPL-eGFP were raised in the dark or in orange light for 16 hours. *ninaE*^{17} + *Rh3* was also illuminated with UV for 16 hours (G). Representative images of the eGFP fluorescence in intact eyes obtained by the water immersion technique are shown. For light-raised *ninaB*^{P315} two individuals are shown that revealed different levels of TRPL-eGFP internalization (I, J). Bar, 15 m. In K, dark-raised flies were transferred to white, blue, green or orange light for 16 hours. Then the percentage of TRPL-

eGFP present in the rhabdomeres was determined. Mean values ± s.d. of at least five experiments are shown.

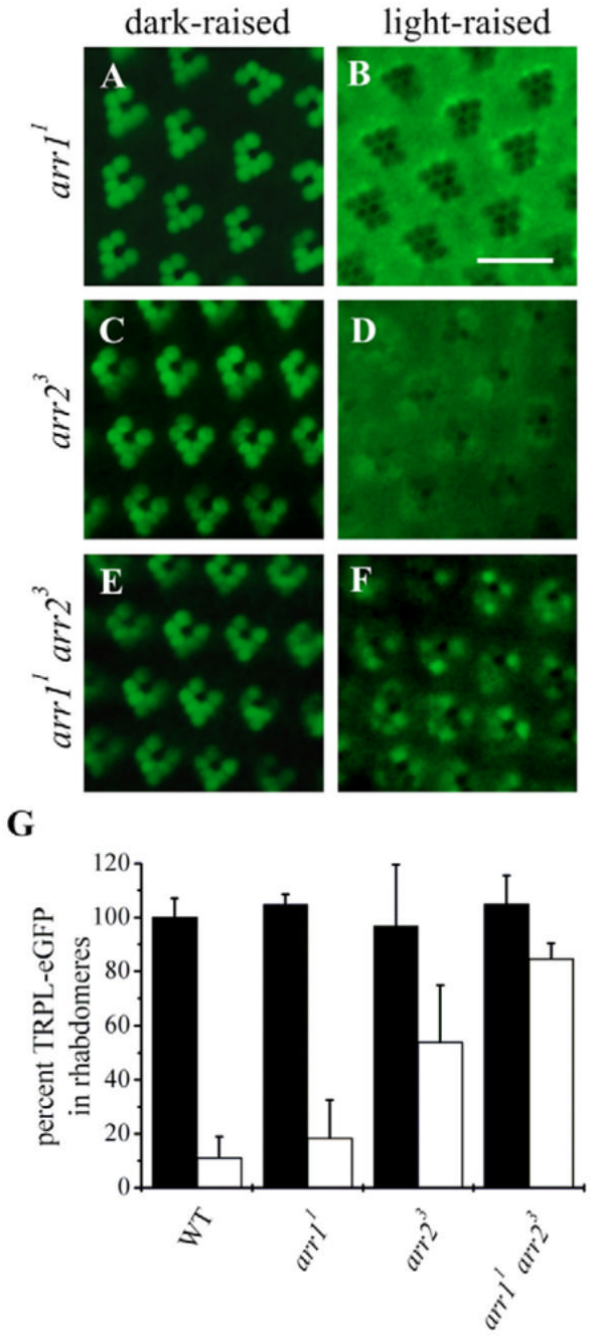

Fig 5.

Role of arrestins in the translocation of TRPL-eGFP. Water immersion images of eGFP-fluorescence in the eyes of the following arrestin mutants raised for 16 hours in the dark or in orange light are shown: (A, B) *arr1*^{1} *cn bw; trpl-eGFP*, (C, D) *w; trpl-eGFP; arr2*^{3} *st*, (E, F) y*w trpl-eGFP; arr1*^{1} *cn bw; arr2*^{3} (*arr1*^{1} *arr2*^{3} double mutant). Bar, 15 m. (G) The rhabdomeral amount of TRPL-eGFP in the dark-raised (black bars) and orange light-exposed (white bars) arrestin mutants was quantified as in Figs 4, 5. For comparison, the relative amount of TRPL-eGFP in the rhabdomeres of dark-raised and orange light-exposed wild-type flies (WT), as determined in Fig. 5, is shown. Values are expressed as a percentage of the R values of dark-raised wild-type flies.

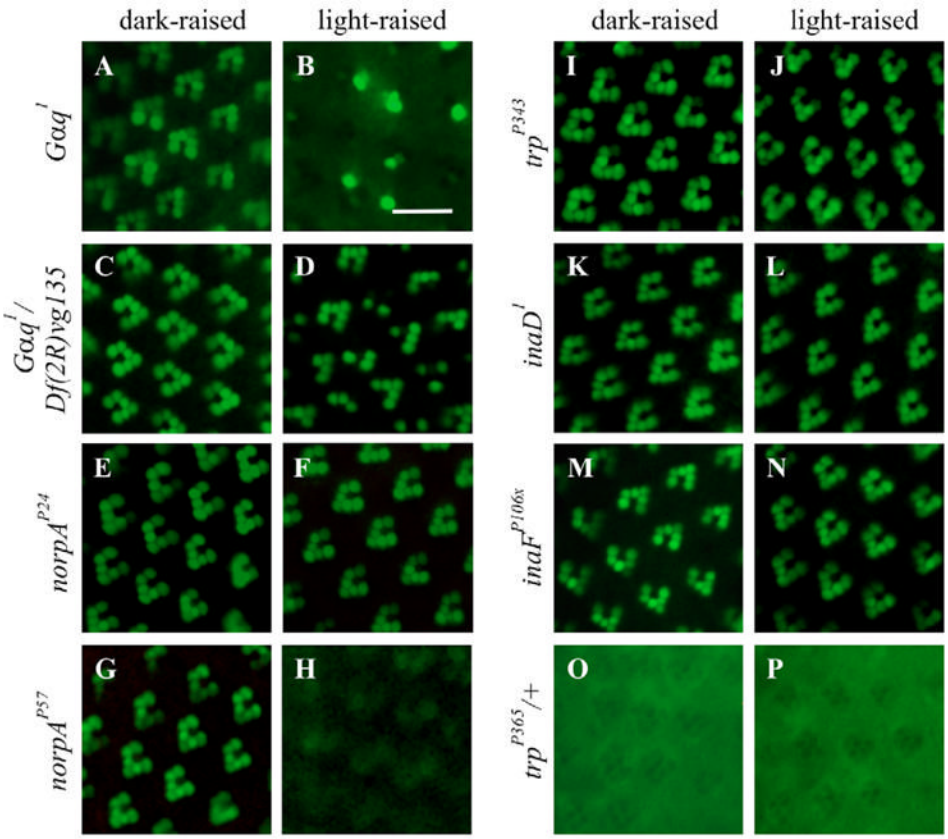

Fig 6.

Components of the phototransduction cascade are required for TRPL-eGFP translocation. Water immersion images of eGFP fluorescence in the eyes of the following mutants raised for 16 hours in the dark or in orange light are shown: (A, B) *w; Gαq^{1}; trpl-eGFP*, (C, D) *w; Gαq^{1}/Df(2R)vg135; trpl-eGFP/+*, (E, F) *w norpA^{P24}; trpl-eGFP/+*, (G, H) *norpA^{P57}; bw/trpl-eGFP; st/+* (note: this mutant has red eyes, hence eGFP-fluorescence in the cell body was shielded by screening pigments), (I, J) *yw; trpl-eGFP; trp^{P343}*, (K, L) *yw; inaD^{1} cn bw; trpl-eGFP*, (M, N) *w inaF^{P106x}; trpl-eGFP/+*, (O, P) *w; trpl-eGFP/+; trp^{P365}/+*. Bar, 15 m.

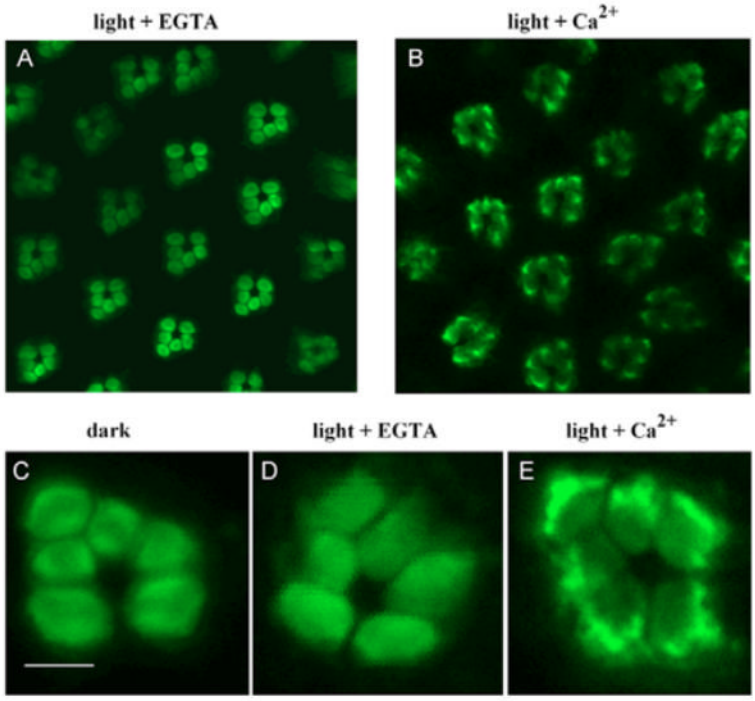

Fig 7.

Removal of extracellular Ca^{2+} inhibited light-induced translocation of TRPL-eGFP. Optical sections of live isolated retinas of flies expressing TRPL-eGFP on a null *trpl* background (*yw; trpl-eGFP trpl*^{302}) are shown. The images were obtained by confocal microscopy using a water immersion objective (Olympus 60×/0.9 w LUMPLan F1). (A, D) Images obtained from sliced heads illuminated for 4 hours (Schott OG 590 unattenuated orange light) and incubated in oxygenated extracellular solution with 1 mM EGTA supplemented with 1% FBS and 5 mM sucrose. (B, E) Images obtained from illuminated sliced heads incubated in oxygenated extracellular solution with 1 mM Ca^{2+} supplemented with 1% FBS and 5 mM sucrose. (C) Image obtained from isolated retina of dark-raised flies. Bars, 10 m (A, B) and 2 m (C–E).

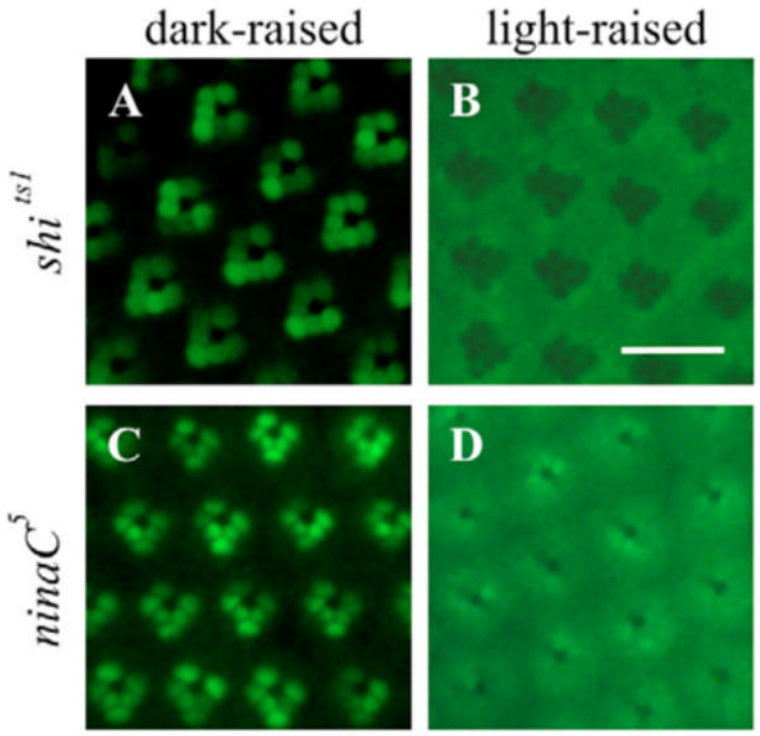

Fig 8.

TRPL-eGFP translocation is independent of dynamin. (A, B) In the temperature sensitive mutant *w shi^{ts1}; trpl-eGFP/+* kept in the dark or in orange light, at a temperature of 29°C localization of TRPL-eGFP resembled that in the wild type. (C, D) In the myosin III mutant *w; ninaC^{5}; trpl-eGFP* endocytosis of TRPL-eGFP but not its translocation from the cell body to the rhabdomere, was partially inhibited. Bar, 15 m.