Dissecting independent channel and scaffolding roles of the *Drosophila* transient receptor potential channel

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**Introduction**

The transient receptor potential (TRP) superfamily is comprised of a large group of related cation channels that function in sensory processes ranging from phototransduction to touch, hearing, taste, olfaction, osmosensation, and thermosensation (for review see Montell, 2005b). As such, dissecting the specific contributions of TRP channels is central to understanding the roles of mammalian TRP proteins in mediating influx of cations, such as Ca$^{2+}$ and Na$^+$. However, in the case of *Drosophila* TRP, which is the founding member of the superfamily that is required for phototransduction (Montell and Rubin, 1989), the protein functions both as a Ca$^{2+}$-permeable channel and as a molecular anchor (Li and Montell, 2000; Tsunoda et al., 2001).

Many of the proteins that are essential for fly visual transduction are organized into a large macromolecular complex, referred to as the signalplex (for review see Montell, 2005a). The molecular scaffold that nucleates the signalplex, inactivation no afterpotential D (INAD; Shieh and Zhu, 1996), consists of multiple PDZ protein interaction modules and appears to be constantly associated with TRP as well as two other important signaling proteins, PLC (encoded by *norpA* [no receptor potential A, encodes PLC]) and PKC (encoded by *inac* [inactivation no afterpotential C, encodes PKC]). These three core binding proteins depend on INAD for localization in the phototransducing compartment of the fly photoreceptor cells, the rhabdomeres (Chevesich et al., 1997; Tsunoda et al., 1997). In addition, at least five other signaling proteins appear to associate with INAD (Huber et al., 1996; Chevesich et al., 1997; Xu et al., 1998; Wes et al., 1999; Goel et al., 2001) and these latter proteins may interact dynamically with INAD. However, none of these noncore binding proteins requires interaction with INAD for normal localization.

A surprising finding is that there is a reciprocal requirement for association of TRP and INAD for concentration of these two proteins in the rhabdomeres (Li and Montell, 2000; Tsunoda et al., 2001). Deletion of the COOH-terminal four residues in TRP destroys the PDZ binding site and results in mislocalization of INAD (Li and Montell, 2000). In turn, the rhabdomeral distributions of PKC and PLC are also disrupted. The interaction between TRP and INAD is not necessary for targeting of these proteins, but rather for subsequent retention in the rhabdomeres. Also, unexpected was the finding that...
interference with the direct interactions between TRP and INAD had no major impact on activation of the TRP channels (Li and Montell, 2000), which in Drosophila photoreceptor cells is very rapid and occurs within milliseconds. These data demonstrate that the TRP channel functions as a molecular anchor, in addition to its more appreciated role as a cation channel.

The dual roles of TRP raise the question as to the impact of altering the channel activity independent of effects on the anchoring function. Null mutations in TRP result in light-dependent retinal degeneration, in addition to causing a transient response to bright light (Cosens and Perry, 1972; Chevesich et al., 1997). Retinal degeneration in fly photoreceptor cells is a common phenomenon that occurs as a result of mutations in nearly any protein important for phototransduction (for review see Pak, 1994; Montell, 1999). However, in most cases the mechanism underlying the retinal degeneration has not been clarified. In some mutants retinal degeneration occurs as a result of formation of stable rhodopsin–arrestin complexes, which in turn lead to endocytosis of rhodopsin (Alloway et al., 2000; Kiselev et al., 2000; Örem and Dolph, 2002). Ca\(^{2+}\) overload due to expression of a constitutively active TRP channel can also lead to rapid cell death in fly photoreceptor cells (Yoon et al., 2000; Wang et al., 2005). However, the mechanism underlying the retinal degeneration in trp-null mutant flies is not known. In particular, it is not clear whether the light-dependent retinal degeneration due to loss of trp function results from disruption of the anchoring role, or from lower Ca\(^{2+}\) influx during light stimulation. This question has not been possible to address because the existing loss-of-function mutations in trp have major impacts on protein levels and consequently disrupt both TRP functions (Montell and Rubin, 1989).

In addition to TRP, there are two related cation channels expressed in photoreceptors, transient receptor potential-like (TRPL), and transient receptor potential \(\gamma\) (TRPY; Phillips et al., 1992; Xu et al., 2000). Currently, there are no loss-of-function mutations in TRPY and elimination of TRPL has only subtle effects on the photoreceptor (Niemyer et al., 1996; Reuss et al., 1997; Leung et al., 2000). Nevertheless, TRPL contributes to phototransduction as flies that are missing both TRP and TRPL are blind (Niemyer et al., 1996; Reuss et al., 1997).

In the current report, we describe the isolation of multiple new trp alleles, including one (trp<P343>) that specifically affected the channel function, but not the molecular anchoring role. In contrast to the wild-type light response, we found that in trp<P343> photoreceptor cells, the light response was transient. This phenotype resulted from a missense mutation in TRPY that juxtaposed to the receptor cells, the light response was transient. This phenotype resulted from a missense mutation in TRPY that was first probed with anti-TRP antibodies and then reprobed with anti-INAD, anti-NORPA, and anti-INAC antibodies. (M) Relative TRP protein levels in trp<sup>P343</sup> flies. The TRP protein level in flies was dark adapted for 2 min before being exposed to a 10-s pulse of orange light. Scale bars are provided indicating the amplitude (mV) and timescale (s). (A–K) Western blots. (A) Western blot was first probed with anti-TRP antibodies and then reprobed with anti-INAD, anti-NORPA, anti-INAC, and anti-tubulin antibodies. (M) Relative TRP protein levels in trp<sup>P343</sup> flies. The TRP protein level in trp<sup>P343</sup> heads was compared with wt, trp<sup>P343</sup> and trp<sup>P343</sup> heterozygotes. The intensities of the bands were determined using a phosphorimager. SDs are indicated.

Results

Identification of new trp alleles

To identify new alleles of the trp locus, we screened the recently isolated collection of chemically induced third chromosome mutations, which display defects in the electoretinogram (ERG) recording (for details see Wang et al., 2005). Exposure of wild-type flies to light results in two discriminable components in the ERG (Fig. 1 A). These include a sustained corneal
negative maintained component arising from responses of all retinal cells (photoreceptor cells and pigment cells) and on- and off-transients emanating from activity in the second-order neurons in the optic lobes (for review see Montell, 1999). The classic trp phenotype is characterized by a transient response to light (Fig. 1 B), resulting from rapid light-dependent inactivation of the remaining TRP cation channel.

We crossed each of the third chromosome mutations to the strong trpP343 allele and identified five that failed to complement the recessive TRP phenotype and therefore represented new trp alleles. Four of the new trp alleles exhibited a transient ERG phenotype indistinguishable from intracellular recordings (Fig. 1, H–J). evident in single photoreceptor cells assayed by performing studies (Fig. 1, C and G; 60% deactivation time: trpP343: 1.5 ± 0.2 s, trpP341: 5.2 ± 1.6 s). The trpP14 phenotype was due to an autonomous defect in the photoreceptor cells, rather than the pigment cells, as the slower decline in the receptor potential was evident in single photoreceptor cells assayed by performing intracellular recordings (Fig. 1, H–J).

Molecular defects and TRP expression in trp alleles

Currently, all of the existing loss-of-function mutations cause large reductions in protein levels, although the molecular lesions have not been defined. Among the extant trp alleles, the one with the strongest phenotype is trpP343. We sequenced the trpP343 genomic region and found that the TRP protein coding region was identical to wild type. Rather, there was a mutation in a conserved 5’ splice site that was essential for mRNA splicing (Table I). The mutation presumably results in instability of the mRNA, as no trpP343 mRNA is detected (Montell and Rubin, 1989). Given the strong phenotype and lack of mRNA or TRP protein expressed in those alleles that displayed a phenotype typical of trp (Fig. 1 K, trp74; 0.2s; 5.2 ± 1.6 s). The trpP14 phenotype was due to an autonomous defect in the photoreceptor cells, rather than the pigment cells, as the slower decline in the receptor potential was evident in single photoreceptor cells assayed by performing intracellular recordings (Fig. 1, H–J).

Table I. Molecular defects in trp alleles

| Allele | Type of mutation | Location | Specific change | TRP protein level |
|--------|------------------|----------|----------------|------------------|
| trP343 | Splice site      | Intron 7; first base of 5’ splice site | GT to AT | ND |
| trpP14 | Two missense     | Pore loop; between TM6 and TRP box 1 | L612F; R671N | 60% wild-type level |
| trP26  | Promoter        | Adjacent to TRP box 1; TRP domain, between boxes 1 and 2 | T674A; W684R | ND |
| trP67  | Two missense    | Fourth ankyrin repeat | ND |
| trP74  | Missense         | Transmembrane domain 1 | G167E | ND |
| trP92  | Deletion/frameshift | Deletion of G in codon 336; stop after adding three residues | ND |

The trp genomic DNA encoding the transcribed region was sequenced for each trp allele. ND, not detected.

1No mutation was found in trpP92, suggesting that there was a mutation affecting expression of the mRNA.

sense mutations. One of these mutations changed an amino acid in the pore loop between transmembrane domains five and six (residue 612; leucine to phenylalanine), whereas the other was situated between the sixth transmembrane segment and a highly conserved sequence referred to as TRP box 1 (residue 671; arginine to glutamine).

Normal anchoring function in trpP14 mutant

Given that TRP has dual functions as a molecular anchor and as a cation channel (Li and Montell, 2000; Tsunoda et al., 2001), we considered whether the TRP phenotype in trpP14 was a consequence of perturbation of the anchoring role. Drosophila compound eyes consist of ~800 repetitive units, referred to as ommatidia, each of which includes six outer photoreceptor cells (R1-6) and a central R7 or R8 cell in the distal region of the retina. Each photoreceptor cell contains a microvillar segment, the rhabdomere, where most of the proteins that function in phototransduction, such as the core members of the signalplex, are concentrated. These include TRP, protein kinase C (INAC), phospholipase C (NORPA), and INAD (Fig. 2 A). Mutations that eliminate or disrupt the anchoring role of TRP result in mislocalization of these core members such that they are present in both the rhabdomeres and cell bodies (Fig. 2 B; Chevesich et al., 1997; Tsunoda et al., 1997; Li and Montell, 2000; Tsunoda et al., 2001). However, other INAD binding partners, such as Rh1, do not depend on TRP for normal localization (Fig. 2 B; Li and Montell, 2000). We found that in trpP14 photoreceptor cells, each of the core and other rhodamal proteins examined displayed a rhabdomere localization pattern indistinguishable from wild type (Fig. 2 C). Consistent with these data, we found that INAD coimmunoprecipitated effectively with the TRP14 protein (Fig. 2 E). These data indicate that the TRP phenotype in trpP14 flies is not due to an alteration...
in the TRP anchoring function. Rather, they raise the possibility that the phenotype is due to a defect in TRP channel function.

**Transient light response in trp14 was due to mutation in trp rather than low levels of expression or mislocalization of TRP**

In *trp* flies such as *trpP343* the transient potential ERG phenotype is a consequence of inactivation of the TRPL channel during constant light stimulation. However, in *trp14* mutant flies, which express TRP but not TRPL, the ERG response is maintained during a typical 5–10-s light pulse (Fig. 1 D). To examine TRP14 channel function independent of TRPL, we introduced the *trp14* allele in a *trpl*-null mutant (*trpl302*) background (Niemeyer et al., 1996). As previously shown, *trpl302; trpP343* flies are blind as they do not express TRPL or TRP (Niemeyer et al., 1996; Reuss et al., 1997). Flies harboring just the *trpl302* mutation show a response to 10 s of light similar to wild type, because these flies express wild-type TRP. In contrast, *trpl302; trp14* flies displayed a TRP phenotype similar to *trpP343* (Fig. 1, F and G; 60% deactivation time: *trpP343* 1.5 ± 0.2 s, *trpP343; trp14*: 5.2 ± 1.6 s, *trpl302; trp14*: 4.2 ± 0.8 s). These data indicate that the transient light response in *trp14* flies was due to disruption of TRP channel function.

To exclude that the *trp14* phenotype was a consequence of mislocalization of TRP, we spatially localized the mutant protein. We performed immunostaining experiments and found that TRP14 was detected exclusively in the rhabdomeres, as was the case for wild-type TRP (Fig. 2, A and C). Therefore, the transient light response in *trp14* was not due to mislocalization of the TRP protein.

Given that *trp14* flies express a 40% lower concentration of TRP, we tested whether a transient light response could be induced by expression of low levels of TRP. Therefore, we generated transgenic flies that expressed varying levels of wild-type TRP under the control of the heat-shock promoter (*hs-trp*) and placed the transgene in a *trpl302; trpP343* genetic background. Even though use of the heat shock protein 70 (*hsp70*) promoter typically results in widespread expression, the TRP protein expressed under the control of the *hsp70* promoter was found exclusively in the retina and not in the optic lobes or elsewhere in the adult head (Fig. 3 A). To induce different low levels of TRP, we exposed the *hs-trp* flies to 30, 60, and 120 min heat-shock treatments, which resulted in the

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**Figure 2. Normal anchoring role of TRP in trp14 flies.** (A–D) Examination of the spatial distributions of INAD and the core INAD binding proteins. The cross sections of compound eyes were obtained from flies 5 d after eclosion maintained under a 12-h light–12-h dark cycle. Sections from flies of the following genotypes were probed with anti-NORPA, anti-INAC, anti-INAD, anti-TRP, and anti-Rh1 antibodies: (A) wild type; (B) *trpP343*; (C) *trp14*; and (D) *calx; trpP343*. (E) Normal interaction between INAD and TRP in *trp14*. Fly head extracts were prepared and immunoprecipitations (IPs) were performed with anti-TRP antibodies. A Western blot containing the IPs was probed with anti-INAD antibodies. The positions of two protein size markers are indicated. (F) A Western blot containing 2% of the total extracts used for the co-IPs was probed with anti-INAD antibodies. The same blot was re-probed with anti-tubulin antibodies. (C) Relative levels of TRP expressed in *trpl; hs-trp; trpP343* flies exposed to different heat shock durations. The TRP protein levels after different heat shock times were compared with the band intensity obtained using 10% as much wild-type extracts. The quantification was performed using a phosphorimager. SDs are indicated. (D–G) ERG recordings obtained in *trpl; hs-trp; trpP343* flies exposed to heat shock treatments of different lengths. Flies were heat shocked for either 0.5, 1, or 2 h at 37°C immediately after eclosion and the ERGs were performed 1 d later by dark adapting the flies for 2 min and exposing them to a 10-s pulse of orange light.
production of ~4%, 7%, and 10% of the wild-type levels of TRP, respectively (Fig. 3, B and C). Expression of only ~10% the normal concentration of TRP restored an ERG in trp14, trpP343 flies, which was not transient (Fig. 3 G). Even 4% the normal levels of TRP did not cause a transient light response similar to trpP343, although the amplitude of the ERG was reduced (Fig. 3 E). These data indicate that the trp14 mutant phenotype was due to the mutation of the TRP14 protein, rather than simply due to expression of low levels or mislocalized TRP protein.

Channel activity critical to prevent light-dependent retinal degeneration

Strong loss-of-function mutations in trp, such as in trpP343, result in light dependent retinal degeneration (Fig. 4). Considering TRP has dual channel and nonchannel roles, disruption of either function could potentially cause retinal degeneration. To address which of these two roles is more critical to prevent retinal cell death, we examined the morphology of trp1272 (Li and Montell, 2000) and trp14 ommatidia, which display specific defects in the scaffold and TRP channel functions, respectively. Wild-type ommatidia contain eight photoreceptor cell rhabdomeres, seven of which are present in any given plane regardless of their age or whether the flies were maintained in the dark or under a light–dark cycle (Fig. 4).

We found that the retinal degeneration in trp14 and trpP343 was much more severe than that in trp1272 flies. In both trpP343 and trp14 flies, the rhabdomeres began to disappear between 7 and 10 d after eclosion and almost no rhabdomeres remained after 14 d of exposure to a 12-h light–12-h dark cycle (Fig. 4, A and B). By contrast most trp1272 flies maintained a full complement of seven rhabdomeres after 14 d of a light–dark cycle (Fig. 4 A). Nevertheless, the size of the rhabdomeres was typically smaller than in similarly aged wild-type and large intracellular vacuoles were present inside the cell bodies indicating that retinal degeneration had initiated (Fig. 4 B). By 30 d after eclosion, most of the rhabdomeres in these flies had degenerated (unpublished data). Thus, the retinal degeneration was much more severe in trpP343 and trp14 than trp1272, indicating that the TRP channel function rather than the scaffold function was more critical to prevent the retinal cell death.

The retinal degeneration resulting from defects in TRP function was suppressed by maintaining the flies in the dark, which keeps the TRP channels in an inactive state. To assess the extent of suppression, we maintained the flies in the dark for 30 d, which was more than twice as long the 14-d light–dark cycle. The mean numbers of rhabdomeres per ommatidium were determined by examining EM cross sections through the distal regions of 80 ommatidia from two separate flies each. Error bars indicate SDs. (B) Examination of the morphology of wt, trp14, trpP343, and trp1272 by transmission EM. Cross sections were obtained from 14-d-old flies kept under a 12 h light–12 h dark cycle. (C) Mean number of rhabdomeres per ommatidium from 30-d-old dark-reared wt, trp14, trpP343, and trp1272 flies examined by transmission EM. Bars, 2 μm.

Rapid retinal degeneration of trp photoreceptors suppressed by mutations in calx and arr2

To explore the mechanism underlying the retinal degeneration in trp mutants, we used a genetic approach. The combination of results described above indicates that a defect in TRP channel function underlies the retinal degeneration in trp flies. If the basis of the retinal degeneration in the trp mutant was due to diminished Ca2+ influx during light stimulation, then the cell death might be reduced by mutations in the Na+/Ca2+ exchanger, CalX, which functions in Ca2+ extrusion in photoreceptor cells.

To test whether calx can suppress the retinal degeneration in trpP343 and trp14, we examined the morphology of calx8, trpP343, and calx8, trp14 compound eyes. As described above, trp14 or trpP343 flies maintained under a light–dark cycle for 14 d displayed nearly complete loss of the rhabdomeres (Fig. 5, A–D). The retinal degeneration in calx8 flies was even
more severe as there were few rhabdomeres left after a 7 d light–dark cycle (unpublished data) and almost no rhabdomeres left after a 14-d light–dark cycle (Fig. 5, A and E). In contrast, most ommatidia from either \(calxB\), \(trpP343\) or \(calxB, trp14\) double mutant flies contained all the rhabdomeres after 14 d under a light–dark cycle (Fig. 5, A, F, and G). Moreover, the core signalplex proteins, NORPA, INAC, and INAD were mislocalized in \(calxB, trpP343\) flies (Fig. 2 D), indicating that introducing the \(calx\) mutation did not prevent loss of the TRP scaffold function in \(trp\). The effect of \(calx\) on \(trp\) was specific as the \(calxB\) mutation did not suppress the cell death resulting from mutations in other phototransduction genes such as \(inaC\), which encodes an eye-enriched PKC (unpublished data). These results indicated that the retinal degeneration in \(trp\) was a consequence of decreased intracellular Ca\(^{2+}\) levels, whereas the photoreceptor cell death in the \(calx\) mutant resulted from Ca\(^{2+}\) overload.

To explore further the mechanism of the retinal degeneration in \(trp\) and \(trp\), we considered whether it could be suppressed by mutations in the gene encoding the major arrestin (\(arrestin2\) [\(arr2\); Dolph et al., 1993]). Elimination of Arr2 reduces the retinal degeneration associated with certain mutations (Alloway et al., 2000; Kiselev et al., 2000), such as \(norpA\) (disrupts phospholipase C), which prevents light-dependent activation of TRP channels. The retinal degeneration in \(norpA\) results from formation of stable rhodopsin–arrestin complexes and subsequent endocytosis of rhodopsin (Orem and Dolph, 2002).

We found that strong mutations in \(arr2\) partially suppressed the retinal degeneration in \(trp\) and \(trp\) flies. Whereas a 14-d exposure to a light–dark cycle resulted in extensive loss of rhabdomeres in \(arr2, trpP343\) or \(trp\) eyes (Fig. 6, B–D), most ommatidia in \(arr2, trpP343\) or \(arr2, trp\) double mutants con-
tially be responsible for the
these mutations were in intriguing positions that could poten-
ture of the region between the sixth transmembrane segment
loop mutation on ion selectivity and the highly conserved na-
respectively; Fig. 7 A). Given the potential effects of a pore–
the L612F or R671Q amino acid substitution (Fig. 7 B). The
transgenic flies expressing TRP isoforms with just
Rh1] promoter and introduced into the
respective; Fig. 7 A). The wild-type or mutant
for 2 min before being exposed to a 10-s pulse of orange light.
The residues in TRPL and TRP
expression of TRP in
ments and the black box stands for TRP box 1. (B) Western blot showing expression of TRP in ninaE-trp, ninaE-trp612F, and ninaE-trp671Q transgenic flies. All three transgenes were in a trp602; trpP343 background. The fly head extracts used for the Western blot were prepared from flies <1 d after eclosion and probed with anti-TRP antibodies. The same blot was reprobed with anti-tubulin antibodies. (C–F) ERG recordings obtained from trp602; trp14 (C) and flies containing the following transgenes in a trp602; trpP343 background: [D] ninaE-trp; [E] ninaE-trp612F; and [F] ninaE-trp671Q. To perform the ERGs, flies <1 d after eclosion were dark adapted for 2 min before being exposed to a 10-s pulse of orange light. We found that the missense mutation juxtaposed to the
TRP domain was responsible for the phenotype in
flies displayed a wild-type ERG response
ground. The Western blot containing head extracts prepared from flies
being exposed to a 10-s pulse of orange light. (C) trp/trp612F; (D) trp/trp671Q; (E–G) The ERGs performed on the following transgenic flies were per-
fused to the
ninaE-trp678Q (trp678Q); and [G] ninaE-trp678R (trp678R). (H) Quantification of the ERG amplitudes obtained in the indicated trp transgenic flies in a trp602; trpP343 background. SDs are indicated.

We found that the missense mutation juxtaposed to the
TRP domain was responsible for the phenotype in trp14. The trpl-
ninaE-trp612F, trpP343 flies displayed a wild-type ERG response (Fig. 7 E) indistinguishable from trpl; ninaE-trp612F, trpP343 (Fig. 7 D). Conversely, the trpl; ninaE-trp671Q, trpP343 flies showed a transient ERG phenotype (Fig. 7 F). Moreover, the trp671Q flies exhibited an ERG phenotype with a more rapid decline typical of
trpP343 suggesting that the 612F mutation resulted in a slight sup-
pression of the TRP phenotype. These data demonstrate that the
missense mutation at residue 671 situated between the sixth
transmembrane domain and TRP box 1 (Montell, 2005b) was the
key mutation responsible for the trp14 phenotype.

Mutation of TRPL in same position as
TRP14 causes channel defect
The residues in TRPL and TRPγ, corresponding to the required arginine 671 in TRP, are also basic amino acids (histidine 678 and arginine 662, respectively; Fig. 8 A) suggesting that a basic residue at this position flanking the TRP domain is essential in
Drosophila TRPC channels. Therefore, we generated trans-

Substitution of a basic residue adjacent to
the TRP domain causes a defect in
channel activity
The two mutations in trp14 alter residues in the pore loop or immediate NH2 terminal to TRP box 1 (residues 612 and 671, respectively; Fig. 7 A). Given the potential effects of a pore-loop mutation on ion selectivity and the highly conserved nature of the region between the sixth transmembrane segment (TM6) and the TRP domain (Montell, 2005b; Fig. 8 A), both of these mutations were in intriguing positions that could potentially be responsible for the trp14 phenotype.

To determine whether one or both mutations were responsible for the transient light response phenotype in trp14, we generated and tested transgenic flies expressing TRP isoforms with just the L612F or R671Q amino acid substitution (trp612F and trp671Q, respectively). The wild-type or mutant trp cDNAs were fused to the ninaE (neither inactivation nor afterpotential E [encodes Rh1]) promoter and introduced into the trp602; trpP343 double mutant background. We subsequently performed Western blots on the transgenic flies demonstrating that wild-type TRP, TRP612F, and TRP671Q were all expressed at similar levels (Fig. 7 B).
genic flies expressing derivatives of TRPL in which histidine 678 was replaced either with a conservative arginine substitution (trpP678R) or with an uncharged glutamine (trpP678Q). The mutant and wild-type trp cDNAs were fused to the ninaE promoter and introduced into a trpH02, trpP343 background. The transgenic TRPL proteins were all expressed at similar levels, though at an approximate sevenfold higher level than in wild-type due to the strong ninaE promoter (Fig. 8 B).

To determine the consequences of the mutations in TRPL, we performed ERGs after introducing the transgenes in a trpH02, trpP242 background. Whereas the double trpH02, trpP241 mutant was blind, overexpression of the wild-type TRPL in this genetic background (trpH41) restored a transient response to light indistinguishable from trpP343 (Fig. 8, C–E). Furthermore, introduction of the trpP678R transgene into the genome of trpH02; trpP341 flies resulted in an ERG response similar to trpH41 (Fig. 8, E, F, and H). Thus, replacing histidine 678 with an arginine did not disrupt TRPL function. However, the amplitude of the ERG was significantly reduced in trpH02; trpP341 flies expressing the trpP678Q transgene with the histidine to glutamine substitution in residue 678 (Fig. 8, G and H). The combination of these results demonstrates a critical role of a basic residue at the corresponding positions in TRP and TRPL, flanking the highly conserved TRP box 1.

Discussion

Drosophila TRP is a multifunctional protein as it serves both as a cation channel and a molecular anchor required for the retention of the scaffold protein, INAD, in the rhabdomeres. The TRP scaffold function is critical because the consequent mislocalization of INAD in turn causes instability and mislocalization of TRP, PLC, and PKC. Thus, over time the core proteins in the signalplex are lost from the rhabdomeres and the visual response is reduced. In addition to TRP, other related proteins may also have dual roles as several vertebrate TRPs, such as gap junctional proteins, maintain a light response, mislocalization of INAD, PLC, and light-induced retinal degeneration. However, it has been postulated that a consequence of decreased light-dependent Ca2+ influx in trp is due primarily to defects in channel function, rather than disruption of the anchoring role. This finding is surprising because elimination of the TRP scaffold function causes time-dependent instability and mislocalization of all four core proteins in the signalplex. Thus, low levels of TRP, INAD, PLC, and PKC result in less pronounced cell death than an amino acid substitution in TRP that disrupts channel function, but has no impact on the concentrations of the core proteins in the signalplex.

The basis for the retinal degeneration was decreased light-dependent Ca2+ influx because the cell death in either trp14 or trp-null mutant flies (trpP343) was greatly reduced by strong loss-of-function mutations in the gene encoding the Na+/Ca2+ exchanger, CalX. This effect was not a consequence of suppression of the anchoring defect because the core signalplex proteins were still mislocalized in calx;trpP343 double mutant flies. Given that the strong light-dependent retinal degeneration in calx was reversibly suppressed by the trpP343 or trp14 mutations, these data also indicated that the cell death in calx resulted from Ca2+ overload.

The mechanism through which decreased Ca2+ influx causes cell death in trp appears to be due at least in part from increased rhodopsin–arrestin complexes. Stable rhodopsin–arrestin complexes and endocytosis of rhodopsin has been associated with degeneration resulting from mutations in the PLC and rhodopsin phosphatase (Alloway et al., 2000; Kiselev et al., 2000; Orem and Dolph, 2002). In the current study, we found that the trp-dependent retinal degeneration was partially suppressed by mutations in arr2. Because Ca2+/calmodulin-dependent phosphorylation of arrestin promotes the release of arrestin from rhodopsin (Alloway and Dolph, 1999), we suggest that a consequence of decreased light-dependent Ca2+ influx in trp14 is reduced phosphorylation of arrestin, which in turn results in increased stability of arrestin–rhodopsin complexes. Alternatively, the reduced Ca2+ influx could result in increased arrestin–rhodopsin complexes due to affects on the rhodopsin.
phosphatase, RDGC (retinal degeneration C). The activity of RDGC is dependent on Ca$$^{2+}$$/calmodulin (Lee and Montell, 2001) and loss of function mutations in rdgC result in stable rhodopsin–arrestin complexes and retinal degeneration (Kiselev et al., 2000).

The observation that decreased TRP-dependent Ca$$^{2+}$$ influx underlies retinal degeneration in fly photoreceptor cells has potential implications in terms of the possible effects on cell survival resulting from loss-of-function mutations in vertebrate TRPs. It appears that constitutive activity of Drosophila and mammalian TRP leads to cell death due to Ca$$^{2+}$$ overload (Yoon et al., 2000; Hara et al., 2002; Wehage et al., 2002; Aarts et al., 2003; Wang et al., 2005). Moreover, constitutive activity of TRPs by anoxic conditions has been proposed to underlie the massive cell death in the mammalian brain that can occur under anoxic conditions, such as occurs as a result of stroke (Agam et al., 2000; Yoon et al., 2000; Hara et al., 2002; Aarts et al., 2003; Wang et al., 2005).

The opposite of constitutive activation is elimination of TRP channel function and whether loss of vertebrate TRP-dependent Ca$$^{2+}$$ influx leads to cell death has not been addressed. However, the results of the current analysis indicate that this is a likely possibility. Elimination TRPM7 from chicken DT40 cells results in cell death (Nadler et al., 2001), but the basis for the requirement for TRPM7 is not known. Given that TRPM7 consists of a TRP channel domain, fused to a COOH-terminal protein kinase domain, the cell death due to loss of TRPM7 could reflect a requirement for either the channel or kinase functions. Moreover, as TRPM7 is highly permeable to both Mg$$^{2+}$$ and Ca$$^{2+}$$ (Nadler et al., 2001), it is unclear the Mg$$^{2+}$$ or Ca$$^{2+}$$ influx is most important for viability. It will be of interest to determine whether the TRPM7-dependent cell death can be suppressed by inhibition of the Na$$^{+}$/Ca$$^{2+}$$ exchanger, as we observed for Drosophila TRP and CalX.

Materials and methods

Fly stocks
The trp$$^{1+},$$ trp$$^{30},$$ trp$$^{50},$$ trp$$^{34},$$ and trp$$^{92}$$ alleles were isolated by performing ethylmethanesulfonate mutagenesis and screening for third chromosome mutations affecting the ERG as we have described recently (Wang et al., 2005). The trp$$^{34}$$ (Montell and Rubin, 1989), calix$$^{4}$$ (Wang et al., 2005), trp$$^{1272}$$ (Li and Montell, 2000), trp$$^{502}$$ (Niemeeyer et al., 1996), and arr$$^{26}$$ (Alloway and Dolph, 1999) mutations were previously described.

Generation of transgenic flies
To express TRP under the control of the hsp70 promoter, the full-length trp cDNA was excised from pBlueScriptK1+ (pBSNot-trp5) with NotI and XbaI and subcloned between the NotI and XbaI sites of pCasseR1s (Thummel and Pirrotta, 1992). To express the wild-type or mutated trp genes under the control of the major rhodopsin (ninaE) promoter, the corresponding cDNAs were subcloned between the NotI and XbaI sites of the pNX vector and the inserts were subsequently excised and introduced into the KpnI site of pCasseR4. To express the wild-type or mutated trpl cDNAs were subcloned into the NotI site of the pNX vector and the inserts were subsequently excised and introduced into the KpnI site of pCasseR4 (Thummel and Pirrotta, 1992). The constructs were injected into w$^{118}$ embryos and transformants were identified on the basis of eye pigmentation. The third chromosome transgenes were selected and put into the trp$^{34}$ background by recombination.

ERG and intracellular recordings
ERG recordings were performed as previously described (Wes et al., 1999). In brief, two glass microelectrodes filled with Ringer’s solution were inserted into small drops of electrode cream, which were placed on the surfaces of the compound eye and the thorax. A light projector (model 765; Newport Corp.) was used for stimulation. The ERGs were amplified with a electrometer (JEOL) and recorded with an analogue-to-digital converter (MacLab/4s) and the Chart v3.4/s program (A/D Instruments). All recordings were performed at room temperature. Intracellular recordings on photoreceptor cells were performed by placing a low-resistance microelectrode filled with 3 M KCl through a small hole on the compound eye and a reference electrode on the thorax as described (Xiong et al., 1994).

Western blots
Fly heads were homogenized in SDS sample buffer with a pellet pestle (Kimble-Kontes) and the proteins were fractionated by SDS-PAGE. The proteins were transferred overnight at 25V to Immobilon-P transfer membranes (Millipore) in Tris-glycine buffer. The blots were probed with the appropriate rabbit or mouse primary antibodies and subsequently with either IgG peroxidase conjugate (anti-rabbit or mouse, respectively; Sigma-Aldrich) or $^{125}$I-labeled protein A (NEN Life Science Products). In those cases in which mouse primary antibodies were used, the blots were probed with rabbit anti–mouse IgG (Sigma-Aldrich) before using the $^{125}$I-labeled protein A. The signals were detected using either ECL reagents (GE Healthcare) or a phosphoimager. Polyclonal rabbit anti-TRP (Chevesich et al., 1997), anti-INAD (Wes et al., 1999), and anti-NORPA antibodies (Wang et al., 2005) were previously described. Monoclonal anti-Rh1 and anti-tubulin antibodies were purchased from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Rabbit anti-TRP antibodies were purchased from Chemicon.

Co-immunoprecipitations
Fly heads (2 mg) were homogenized in 200 $\mu$L of buffer A (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl$_2$, 10% sucrose, 1 mM EDTA, and complete protease inhibitor (Boehringer, 1997) containing 1% CHAPS with a pellet pestle. The extracts were centrifuged at 15,000 g for 5 min at 4°C to remove cellular debris. Homogenates were diluted with 800 $\mu$L buffer A to achieve a final concentration of 0.2% CHAPS. 1 $\mu$L of anti-TRP antibodies were added and incubated for 1 h at 4°C, followed by 10 min centrifugation at 4°C. To reduce nonspecific binding, the protein A–Sepharose beads (GE Healthcare) were first incubated with buffer A containing 0.2% CHAPS and 1% BSA for 1 h at 4°C. The immunocomplexes were then incubated for 30 min at 4°C with 50 $\mu$L of a 50% slurry of the blocked protein A–Sepharose beads in buffer A with 0.2% CHAPS, followed by 1 min centrifugation at 5,000 g to pellet the protein A–Sepharose beads and associated immunocomplexes. After washing twice with buffer A, the beads were resuspended in SDS sample buffer, fractionated by SDS-PAGE, and a Western blot was probed with anti-INAD antibodies.

Immunolocalizations
Hemisected fly heads were fixed with paraformaldehyde and embedded in LR White resin as described previously (Porter and Montell, 1993). Cross sections (0.5 $\mu$m) of compound eyes were cut through the distal region of the retina, which included the R7 cells, and stained with primary antibodies (1:200) and FITC-labeled secondary antibodies (1:200) as described previously (Porter and Montell, 1993). Samples were examined with a Zeiss microscope (Carl Zeiss Microimaging, Inc.) using a Plan-Apochromat 63× objective and the images were acquired using a SensiCam camera ( Cooke) and IPLab 3.6.5 software. The images were then transferred into Adobe Photoshop 7.0 for assembling the figures.

Transmission EM
Heads were dissected from flies reared under a 12-h light–12-h dark cycle or in constant darkness and embedded for transmission EM as described previously (Porter et al. 1992) except that 0.1 M sodium phosphate (pH 7.4) was used as the buffer. Thin sections (85 nm) prepared at a depth of 30 $\mu$m were examined by transmission EM using an electron microscope (FEI Tecnai 12; Carl Zeiss Microimaging, Inc.). The images were acquired using a camera (model 794; Gatan) and Digital Micrograph software (Gatan, Inc.) and converted into tiff files.

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