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The Drosophila Light-Activated Conductance Is Composed of the Two Channels TRP and TRPL

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Summary

Drosophila phototransduction is a G protein–coupled, calcium-regulated signaling cascade that serves as a model system for the dissection of phospholipase C (PLC) signaling in vivo. The Drosophila light-activated conductance is constituted in part by the *transient receptor potential* (*trp*) ion channel, yet *trp* mutants still display a robust response demonstrating the presence of additional channels. The *transient receptor potential-like* (*trpl*) gene encodes a protein displaying 40% amino acid identity with TRP. Mammalian homologs of TRP and TRPL recently have been isolated and postulated to encode components of the elusive I_{crac} conductance. We now show that TRP and TRPL localize to the membrane of the transducing organelle, together with rhodopsin and PLC, consistent with a role in PLC signaling during phototransduction. To determine the function of TRPL in vivo, we isolated *trpl* mutants and characterized them physiologically and genetically. We demonstrate that the light-activated conductance is composed of TRP and TRPL ion channels and that each can be activated on its own. We also use genetic and electrophysiological tools to study the contribution of each channel type to the light response and show that TRP and TRPL can serve partially overlapping functions.

Introduction

The cellular responses to a wide variety of hormones, neurotransmitters, and sensory input are mediated by G protein–coupled receptors that signal via phosphoinositides and their breakdown products. In Drosophila photoreceptor neurons, light activation of rhodopsin activates a $G_{q\alpha}$ that in turn activates a phospholipase C (PLC). PLC catalyzes the breakdown of the minor membrane phospholipid phosphatidylinositol bisphosphate (PIP_2) into the two intracellular messengers inositol triphosphate (IP_3) and diacylglycerol (DAG); these then lead to the eventual opening (and modulation) of membrane channels and the generation of a receptor potential (reviewed in Ranganathan et al., 1995; Zuker, 1996). Many of the components involved in the activation and regulation of this signaling pathway have been identified, and this cascade has served as a model system for the genetic dissection of G protein–coupled, PLC-signaling pathways in vivo (Zuker, 1996). These genetic studies have led not only to the isolation and characterization of genes encoding proteins expected to participate in this process, like rhodopsin (Rh1; O'Tousa et al., 1985; Zuker et al., 1985), G protein subunits ($DG_{q\alpha}$ and $G\beta e$; Lee et al., 1990; Dolph et al., 1994; Scott et al., 1995), and PLC (NorpA; Bloomquist et al., 1988), but also to a number of proteins whose involvement could not have been predicted on biochemical grounds, like the gene products of *ninaA* (Larrivee et al., 1981; Stamnes et al., 1991; Baker et al., 1994), *ninaC* (Montell and Rubin, 1988; Porter and Montell, 1993; Porter et al., 1993), *eye-cds* (Wu et al., 1995), *inaD* (Pak, 1979; Shieh and Niemeyer, 1995), and *rdgB* (Harris and Stark, 1977; Vihtelic et al., 1991).

Perhaps one of the most important questions remaining in invertebrate phototransduction is the nature of the light-activated channels and their mechanism of gating. Since the effector of this signaling pathway is a PLC_β, the light-activated conductance has been hypothesized to be composed of ion channels responding to IP_3, calcium, or to a capacitative-calcium entry signal (also known as store-operated-calcium entry) (Hardie and Minke, 1993; Ranganathan et al., 1995). This latter class would be the functional homolog of the vertebrate I_{crac} channel (calcium-release-activated channel), a conductance of unknown molecular composition essential to maintain proper calcium homeostasis and refilling of the intracellular calcium stores in a wide range of cells and tissues (Hoth and Penner, 1992; Berridge, 1995). I_{crac} is believed to be gated either by a diffusible messenger released upon emptying of the stores (Parekh et al., 1993; Randriamampita and Tsien, 1993), or by direct protein–protein interaction with the IP_3 receptor in the endoplasmic reticulum, much like has been postulated for the dihydropyridine and ryanodine receptor in skeletal muscle (Irvine, 1990; Berridge, 1995). In such a model, light-activation of rhodopsin would result in the production of IP_3, which would lead to the release (and emptying) of calcium from IP_3-sensitive internal stores and the subsequent gating of plasma membrane channels, either via conformational coupling or a diffusible signal.

Genetic and electrophysiological experiments have shown that the light-activated conductance in Drosophila photoreceptors is composed of at least two distinct ion channels, one of which is encoded by the *trp* gene (Hardie and Minke, 1992). *trp* encodes a 145 kDa protein with structural similarity to vertebrate voltage-gated calcium channels (Montell and Rubin, 1989; Phillips et al., 1992). However, the alternating positive arginine residues in the S4 domain that act as voltage sensors are absent in TRP. This is consistent with the view that the light-activated channels are gated by an intracellular signal and not by changes in membrane potential. Drosophila mutants defective in TRP are unable to sustain a steady-state current during prolonged light stimulation; this leads to premature termination of the light-activated conductance and to transient inactivation (thus the

name transient receptor potential) (Cosens and Manning, 1969; Minke et al., 1975). Since intracellular calcium is necessary to maintain photoreceptor responsiveness (Hardie et al., 1993), it has been hypothesized that the *trp* mutant phenotype results from a depletion of internal calcium stores during prolonged light stimuli. In wild-type photoreceptors, these stores would be rapidly refilled by opening of the TRP channel (Minke and Selinger, 1992). In accord with this model, the TRP channel has been shown to be the primary carrier of calcium during the light response (Peretz et al., 1994a, 1994b), and extracellular application of the calcium channel blocker lanthanum mimics the *trp* phenotype in wild-type photoreceptor cells (Hochstrate, 1989). *trp* mutants still display a response to light stimuli (Minke et al., 1975; Hardie and Minke, 1992), demonstrating that the light-activated conductance cannot be composed solely of TRP channels. This finding has complicated the analysis of TRP function (because there are no mutants that only express the TRP channel) and of the light-activated conductance, and has hindered the identification of the final messenger responsible for triggering activation of the light response. To determine the nature of the light-activated conductance, we set out to identify the additional component(s) and to carry out a genetic and physiological dissection of its function. We now show that TRPL, a photoreceptor-specific TRP homolog, encodes the additional component and that together TRP and TRPL mediate the light response of this PLC-based signaling cascade.

Results and Discussion

Isolation of *trpl* Mutants

TRPL is a visual system–specific TRP homolog displaying 40% amino acid sequence identity with TRP (Phillips et al., 1992). Like TRP, it shares structural similarity to voltage-activated calcium channels. Recently, Schilling and coworkers showed that expression of TRPL in heterologous cells results in the appearance of a nonselective cation conductance (Hu et al., 1994; Dong et al., 1995; Hu and Schilling, 1995). Given its tissue specificity and its ability to function as an ion channel, we set out to determine whether *trpl* encodes a component of the light-activated conductance. Our approach was to screen for Drosophila mutants defective in this gene. A serious obstacle in setting up such a screen was the possibility that *trp* and *trpl* may serve partially overlapping functions. Because *trpl* is expressed specifically in the visual system, mutations in this gene should have no effect on viability. Therefore, we used a screening strategy that was based on the loss of TRPL antigen on immunoblots rather than on a hypothetical physiological or behavioral defect (see Dolph et al., 1993). In essence, we generated fly stocks containing individual homozygous mutagenized second chromosomes (*trpl* maps to the second chromosome at position 46B$^{1-2}$) and each stock was then subjected to immunoblot analysis for the loss of anti-TRPL immunoreactivity. Analysis of 1093 lines yielded one allele, *trpl*^{302}.

Using the polymerase chain reaction we isolated the *trpl*^{302} gene and determined its entire nucleotide sequence. *trpl*^{302} has an amber nonsense codon at position 302, leading to premature termination of the polypeptide chain before the first transmembrane segment; this represents a complete null allele. Figure 1 shows a Western blot demonstrating the absence of TRPL protein in *trpl*^{302} mutants. This mutation did not affect the expression of TRP, or of a number of other molecules involved in the phototransduction cascade (e.g., Rh1, eye-PKC; data not shown).

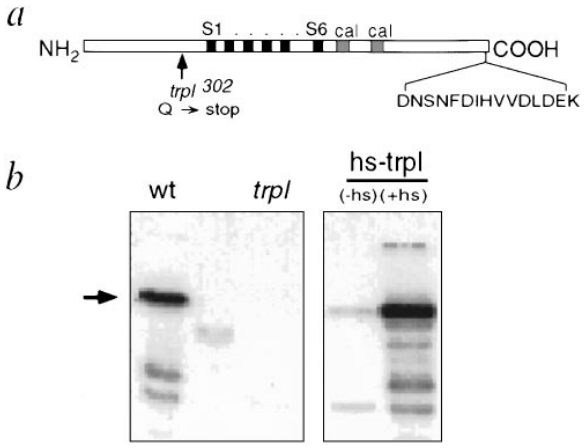


Figure 1. *trpl*^{302} Is a Null Allele

(a) Shown is a diagram of the *trpl* gene structure. S1-S6 represent the putative transmembrane domains and the shaded boxes denote calmodulin-binding domains (Phillips et al., 1992). The position of the amber stop codon in the *trpl*^{302} mutant is indicated by the arrow. Shown in expanded form is the region used to generate the C-terminal anti-peptide antibody.

(b) TRPL expression was examined in wild-type, *trpl*^{302} mutant, and transgenic flies expressing the *trpl* gene under the control of a heat shock–inducible promoter. Shown is a Western blot demonstrating the complete absence of TRPL protein in mutant flies. The right gel shows that a 1 hr heat shock pulse (37°C) is sufficient to induce expression of TRPL in *trpl*; P[hs-trpl] transgenic flies (see Experimental Procedures). This genetic background was used in all of the transformation rescue experiments. The small amount of protein in the uninduced lane is due to leakiness of the heat shock promoter.

TRP and TRPL Localize to the Rhabdomeres

If TRP and TRPL are involved in phototransduction, then these proteins should localize either to the rhabdomeres (the specialized microvillar organelles containing the visual pigment rhodopsin and most of the proteins involved in phototransduction) (Wolff and Ready, 1993), or close to the subrhabdomeral cisternae (SRC; the internal stores of calcium found at the base of the rhabdomere). Recently, Pollock et al. (1995) used an anti-TRP monoclonal antibody and indirect immunofluorescent staining of fly retinas to show that TRP localizes to the base of the rhabdomeres. These findings have been used as experimental support for the suggestion that TRP functions as a store-operated channel and serves to refill the internal calcium stores utilizing a capacitative entry model via protein–protein interaction (conformational-coupling) with a receptor in the SRC (Hardie and Minke, 1993). To further determine the subcellular distribution of TRP and TRPL, we generated polyclonal antibodies to TRP and TRPL and used them in indirect immunofluorescence stainings of 1 μm thick cross sections of wild-type and mutant retinas (see Experimental Procedures

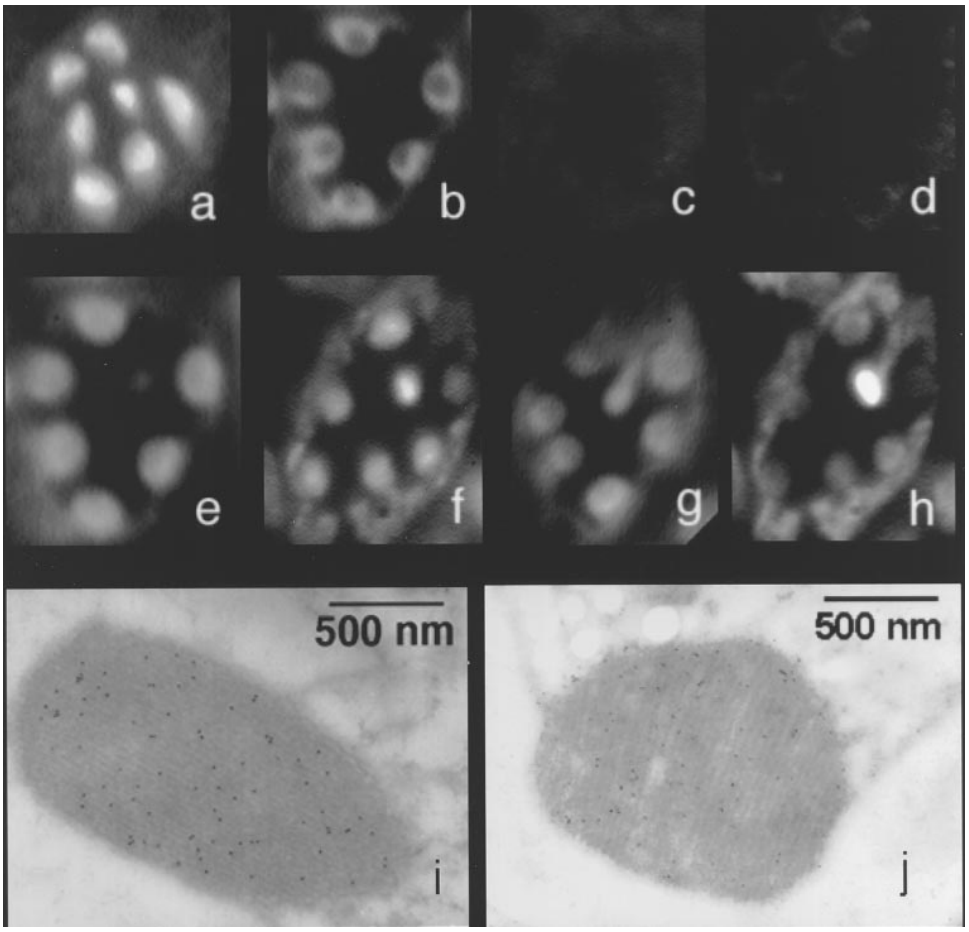


Figure 2. TRP and TRPL Are Expressed in the Rhabdomeres

(a–h) Indirect immunofluorescence staining of 1 μm thick cross sections of wild-type and mutant photoreceptors.

(a) wild-type (wt) cells stained with anti-PLC (NorpA) demonstrating localization of the protein to the rhabdomeres.

(b) wt cells stained with anti-Rh1 showing restricted expression in the rhabdomeres of R1–R6 photoreceptor neurons (they are the only ones that express Rh1 rhodopsin).

(c and d) *trp* and *trpl* mutants stained with anti-TRP and anti-TRPL, respectively. Note the absence of staining in the corresponding mutant background.

(e and f) TRP is primarily expressed in the rhabdomeres. Shown are sections from (e) wild-type and (f) *norpA* photoreceptors using anti-TRP antibodies.

(g and h) the TRPL protein also localizes to the rhabdomeres in *trp* and *norpA* mutants.

(f) and (h) were double stained for TRP and TRPL, demonstrating coexpression of both proteins (the R7 cells label prominently in *norpA* mutants).

(i and j) EM immunogold localization of TRP and TRPL showing expression of both proteins in the microvillar membranes of the rhabdomeres (see Experimental Procedures for details). Both TRP and TRPL also label intracellular vesicles (data not shown), but neither show preferential localization to the base of the rhabdomeres or the SRC.

for details on the antibodies). We also carried out ImmunoEM staining using gold-conjugated secondary antibodies. Our results (Figure 2) demonstrate that both TRP and TRPL localize to the rhabdomeres. Interestingly, using an antibody directed against the last 15 residues of TRPL, we cannot detect significant labeling in the rhabdomere unless we first unmask the epitope using secondary mutations that lead to disruption of the signaling complexes in the rhabdomeres (e.g., *ninaE*, *norpA*, and *trp*). TRPL has been independently localized to the rhabdomeres of wild type cells by using a collection of monoclonal antibodies directed against TRPL fusion proteins (C. Montell, personal communication).

Our localization of TRP to the rhabdomeres, using two independent polyclonal antibodies (Figures 2e and 2f and data not shown), differs from the results of Pollock et al. (1995) in that we do not detect preferential labeling at the base of the rhabdomeres even when using immunoelectron microscopy. We believe this discrepancy is due to their use of a single monoclonal antibody, which raises the distinct possibility that the epitope is masked in the rhabdomeres, and their use of newly eclosed flies as opposed to mature flies. Kumar and Ready (1995) and we (B. N. and C. Z., unpublished data) have recently shown that rhabdomeres have not fully developed in late pupae or newly eclosed flies, and that rhodopsin, a membrane protein normally found exclusively in the rhabdomeres of mature flies localizes to the base of the

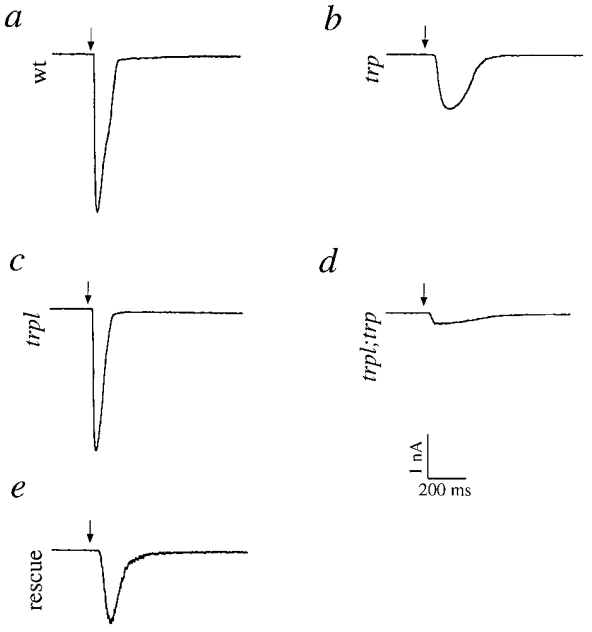


Figure 3. Photoreceptor Cell Function in *trp* and *trpl* Mutants

Shown are representative whole-cell, voltage-clamped recordings of light-activated currents from (a) wild-type controls, (b) *trp*, (c) *trpl*, (d) *trpl; trp*, and (e) *trpl* P[hs-trpl+]; *trp* (rescue) photoreceptors. Cells were stimulated with 10 ms flashes of 580 nm light (log (I) = −2) at a holding potential of −60 mV at the time indicated by the arrow. Note the dramatic loss of responsiveness in the double mutant. Recordings in (e) were performed 3–4 hr after heat shock–induced expression of the P[hs-trpl+] transgene (1 hr at 37°C; note that only the *trpl* component of the phenotype should be rescued).

rhabdomeres at these early stages (possibly in transit to the microvillae). Our localization of TRP and TRPL to the rhabdomeres is consistent with a role for both proteins in phototransduction, and raises serious doubts for a model invoking activation of TRP by direct protein–protein interaction with a partner protein in the SRC (Hardie and Minke, 1993; Pollock et al., 1995).

TRPL Is an Essential Component of the Light-Activated Conductance

To determine whether *trpl* mutants have a defect in their signaling properties, we used whole-cell patch-clamp recordings to analyze in detail the electrophysiological responses of *trpl* mutant photoreceptors. The light responses of wild-type, *trp* and *trpl* mutants are shown in Figure 3. As expected, *trp* mutants (Figure 3b; Table 1) display responses with reduced amplitudes and defective kinetics (Hardie and Minke, 1992). The approximately 10-fold reduction in light sensitivity seen in *trp* mutants (Figure 4a) and the corresponding shift in reversal potential (Figures 4b and 4c) is consistent with a model in which *trp* encodes the major fraction of the light-activated channels (see below). The slow kinetics are consistent with the reduction in calcium entry expected from the loss of the major calcium entry pathway and the requirement for calcium in positive and negative feedback (Hardie, 1991; Ranganathan et al., 1991; Peretz et al., 1994a ,1994b). Surprisingly, *trpl* mutants are nearly indistinguishable from wild-type controls both in sensitivity and response kinetics (Figures 3a, 3c, and 4; Table 1). We reasoned that the lack of a phenotype in *trpl* mutants may be due to the presence of functional TRP channels masking its requirement. This is particularly relevant since TRP carries a large fraction of the light-activated conductance (compare Figures 3a and 3b), and a loss of the remaining component may not be easily revealed in a trp^+ background. If this is true, then a *trpl; trp* double mutant should have a dramatic loss of responsiveness. Indeed, Figures 3d and 4a show that *trpl; trp* double mutants have greater than a 10,000-fold loss of sensitivity. This loss is due to the lack of TRPL in a *trp* background since reintroduction of the wild-type $trpl^+$ gene into the *trpl; trp* mutant hosts by P element–mediated germ-line transformation rescues the defect and restores visual physiology (compare Figures 3d and 3e). These results demonstrate three important aspects of TRP and TRPL function in vivo. First, TRPL activity is masked by functional TRP channels, and together they are essential components of the light-activated conductance. Second, TRP and TRPL need not form heteromeric channels because *trpl* mutants only contain TRP and *trp* mutants only have TRPL (but see next section). Third, since *trp* and *trpl* mutants respond to light-activation, then each channel on its own must be capable of sensing the intracellular messenger that gates the light-activated conductance. Furthermore, activation of one does not require prior activation of the other.

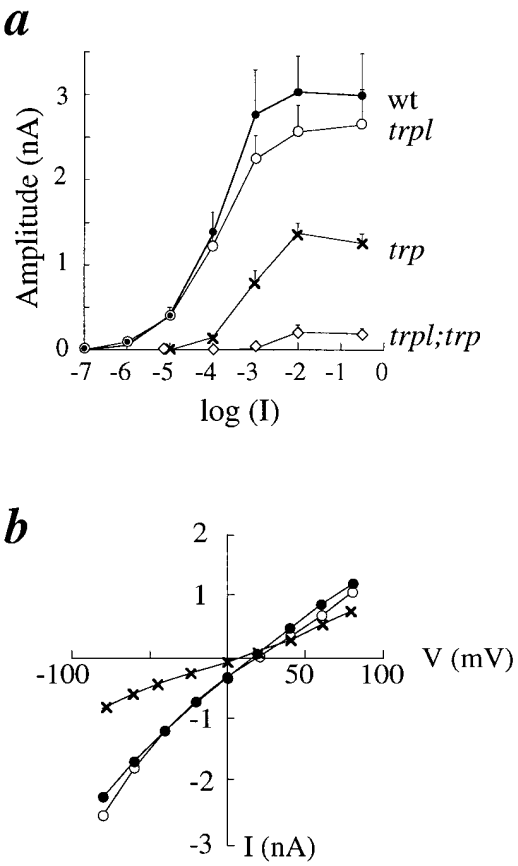
TRP and TRPL Ion Channels Make Up the Light-Activated Conductance

The small current remaining in the *trpl; trp* double mutant (Figure 3d) could be due to the presence of a third light-activated channel, or to small amounts of functional TRP or TRPL protein remaining in the mutant flies. While $trpl^{302}$ is a complete null allele (see Figure 1), the nature of

Table 1. Response Kinetics of *trp* and *trpl* Photoreceptors

	Latency (ms)	Rise Time (ms)	Decay Time (ms)	Amplitude (pA)
wt	17 ± 0.84	14 ± 1.3	76 ± 7.0	3544 ± 470
trpl	16 ± 0.76	14 ± 1.3	56 ± 3.4	3091 ± 377
trp	28 ± 1.2	23 ± 2.1	104 ± 13.6	1376 ± 121
trpl; *trp*	41 ± 6.6	89 ± 19.9	274 ± 65.3	234 ± 83
Rescue	33 ± 2.4	41 ± 6.7	190 ± 32.0	1274 ± 388

Shown are values (± SEM) for latency (defined as the time elapsed from the onset of the light stimulus to the beginning of the response), rise time (calculated as the time between 10% and 90% of peak response amplitude), decay time (calculated as the time between 10% and 90% deactivation), and peak amplitude. In all cases at least six cells from five different flies were analyzed.



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Genotype	Erev (mV)
wt	21.2 ± 5.5
trp	4.8 ± 5.1
trpl	17.0 ± 3.5
trpl; trp	19.5 ± 1.1
rescue*	3.3 ± 3.1

Figure 4. Characterization of *trp* and *trpl* Physiology

(a) Current amplitudes (± SEM) as a function of light intensity. Both wild-type (n = 8) and *trpl* (n = 9) photoreceptors show a sigmoidal dependency of response amplitudes with light intensity and reach saturation at log[I] = −2. *trp* mutants show a 10-fold loss of sensitivity and maximal amplitudes that are 1/3 of those of wild type (n = 10). *trpl; trp* double mutants display a much more severe reduction in sensitivity and peak amplitudes that are ~1/20 of wild type (n = 6).

(b) Current–voltage relationships in wt, *trp*, and *trpl* mutants. Shown are sample traces; like in panel (a) closed circles = wild type, open circles = *trpl*, and crosses = *trp*. Note the shift in *trp* and the match between wild type and *trpl*.

(c) Reversal potential values (E_{rev} ± SD; n = 3 for *trp* and rescue, n = 5 for all others) in the different genetic backgrounds. Note that rescue refers to *trpl*, P[hs-trpl]; *trp* flies, so they should now look like *trp* mutants. Other ionic conditions under which reversal potentials were measured included: bath solution 120 mM N-methyl-D-glucamine, 10 mM CsCl, and either 1.5 mM $CaCl_2$ or 10 mM $BaCl_2$ with internal solution containing 120 mM CsCl (data not shown, see text).

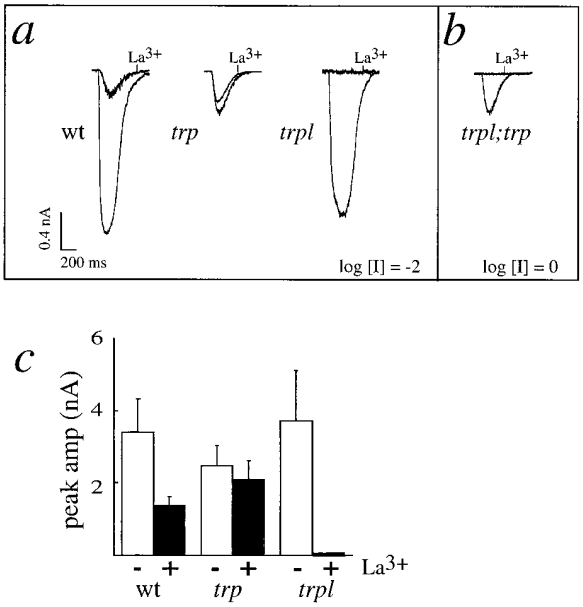

Figure 5. Application of Lanthanum to *trpl* Mutants Abolishes the Light Response

Traces of light-induced currents in (a) wt, *trp*, and *trpl* photoreceptors, and (b) a *trpl; trp* mutant are shown before (larger responses) and after (smaller responses) addition of lanthanum to the extracellular bath (final concentration of 10 μm). Note that the light response in *trpl* and *trpl; trp* cells is eliminated after addition of lanthanum. Cells were stimulated with a 10 ms flash of 580 nm light of log[I] = −2 in (a) and log[I] = 0 in (b) at 50 ms after the onset of the trace.

(c) Average peak amplitudes of light responses in the absence (open bars) and presence (closed bars) of 10 μm lanthanum in the bath. Mean amplitudes ± SEM are for wt (n = 6): 3400 ± 900 (− La^{3+}) and 1360 ± 250 (+ La^{3+}); for *trp* (n = 6): 2460 ± 570 (− La^{3+}) and 2100 ± 510 (+ La^{3+}); and for *trpl* (n = 6): 3750 ± 1400 (− La^{3+}) and 0 (+ La^{3+}). Responses were induced by a 10 ms flash of 580 nm light of log[I] = −1. For *trpl; trp* (n = 5), mean amplitudes were 250 ± 67 (− La^{3+}) and 0 (+ La^{3+}) after stimulation by a 10 ms flash of 580 nm light of log[I] = 0 (data not shown).

the mutation in trp^{301} (the strongest *trp* allele, expressing less than 1% of wild-type levels of protein) is not known. To distinguish between these two alternatives, we examined the electrophysiological properties of the remaining current using two independent strategies. First, the calcium channel blocker lanthanum at 10 μM strongly inhibits the TRP channel (Hochstrate, 1989; Hardie and Minke, 1992), but has little or no effect on TRPL: wild-type photoreceptors treated with lanthanum look very much like *trp* mutants, but *trp* mutants (which have a normal TRPL channel) treated with lanthanum are largely unaffected (Figures 5a and 5c). Therefore, if the residual current in the *trpl; trp* mutants is due to a small amount of functional TRP channel, adding lanthanum to the double mutant should result in a complete loss of the light response. Indeed, Figure 5b demonstrates this is the case. Furthermore, when we expose *trpl* mutants, which presumably only have functional TRP, to lanthanum we now eliminate the light response (these cells have a complete loss of TRP and TRPL function) (Figures 5a and 5c). Second, we analyzed the reversal potential (E_{rev}) of the small conductance remaining in the double mutant. *trp* mutants display a

significant shift in E_{rev} due to the loss of the major, calcium-selective conductance (Figures 4b and 4c) (Hardie and Minke, 1992). In contrast, E_{rev} in *trpl* mutants is not significantly different from that in wild-type photoreceptors even under a wide range of ionic conditions (see legend of Figure 4b). This is not unexpected since the loss of a minor nonselective channel is likely to be masked by the major selective channel. However, if the small conductance recorded in the double mutant is due to remaining functional TRP channels, then its E_{rev} should now approach that of *trpl* (~17 mV) and not that of *trp* mutants (~5 mV) as the only channel present would be a small amount of TRP. As predicted, the E_{rev} of the double mutant (~20 mV) matches that of *trpl* (~17 mV) (Figure 4c). Furthermore, the E_{rev} of double mutant photoreceptors expressing a TRPL transgene now shifts back down to ~3 mV. Together, these genetic and electrophysiological studies demonstrate that TRP and TRPL comprise the light-activated conductance.

Single Photon Responses of *trp* and *trpl* Mutants

In wild-type photoreceptors single photons give rise to unitary events known as quantum bumps (Yeandle, 1957; Baylor et al., 1979; Stieve, 1986). A mechanistic interpretation of this phenomenon is that activation of a single rhodopsin molecule leads to the production of a threshold amount of final messenger, which in turn leads to the opening of a number of ion channels. In invertebrate photoreceptors, quantum bump amplitudes vary greatly (Figures 6a and 6b) (Stieve, 1986); this reflects the inherent variability in the amplification process in these cells. Mutants that affect steps upstream of PLC activation do not change bump shape, but affect bump frequency (reviewed in Pak et al., 1976; see also, Scott et al., 1995). This can be easily rationalized by understanding that the activation of a rhodopsin molecule either does or does not produce the threshold level of messenger that then leads to the generation of a bump. Therefore, defects upstream of PLC affect frequency rather than amplitude by reducing the efficiency with which the threshold of messenger is produced. In contrast, one would predict that a reduction in the amount of light-activated channels should have a dramatic effect on the mean bump amplitudes. Thus, we examined quantal responses in wild-type, *trp*, *trpl*, and *trpl; trp* mutant photoreceptors and determined quantum bump amplitudes and kinetics.

Previous studies relying on noise analysis suggested that the size of quantum bumps in *trp* mutants is similar to wild-type cells (Minke et al., 1975; Barash et al., 1988). However, if TRP represents the major light-activated channel, *trp* mutants would be expected to have a dramatic reduction in bump amplitude. Figure 6 shows that *trp* photoreceptor cells produce bumps with mean amplitudes that are <30% of wild type. This result, together with the data presented before, formally demonstrates that *trp* encodes the major component of the light-activated conductance.

In our analysis of quantum bumps, we could detect no overt phenotype in *trpl* mutants (Figure 6). However, if *trpl* encodes the minor component of the light-activated conductance, the broad distribution of quantum bump size and shape seen in wild-type cells would likely obscure small differences. We reasoned that if TRP and TRPL make up the light-activated conductance, quantum bump amplitude should be massively reduced in the double mutant. As predicted, analysis of quantum bumps reveal a dramatic bump phenotype in *trpl; trp* mutants (Figures 6a and 6b): bump amplitudes are <1/30 of wild type cells. These results firmly establish a role for TRP and TRPL in the generation of a quantum bump and independently demonstrate their overlapping function.

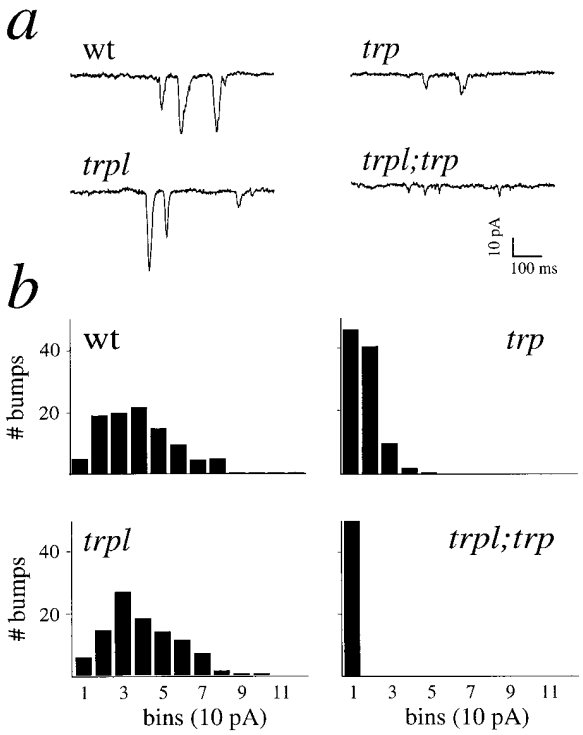

Figure 6. *trp* and *trpl* Contribute to the Size of Single Photon Responses

(a) Examples of quantum bumps induced by 580 nm light of log[I] = −7 for wt, *trp*, and *trpl* photoreceptors. *trpl; trp* photoreceptors show detectable currents at log[I] = −5.

(b) Amplitude histograms demonstrate that the mean bump amplitude is notably smaller in *trp* and dramatically smaller in *trpl; trp* cells. Response amplitudes were measured for 220 wt bumps (13 cells), 203 *trp* bumps (7 cells), 213 *trpl* bumps (13 cells), and 50 *trpl; trp* bumps (5 cells). Quantum bumps were generated by 10 ms flashes of 580 nm light of log[I] = −6.5 for wt, *trp*, and *trpl* and log[I] = −4.5 for *trpl; trp* so that the probability of a quantum bump occurring was ~50%.

Concluding Remarks

Although much progress has been made in identifying the components that make up the Drosophila phototransduction cascade in the past 15 years, the nature of the ion channels and their gating mechanism has remained elusive. The dissection of this final step of phototransduction has been hampered by the inability to carry out electrophysiological recordings on isolated membrane patches containing the light-activated channels, as was done for the cGMP-gated conductance of vertebrate photoreceptors (Fesenko et al., 1985). This

is due to the inaccessibility and architectural complexity of rhabdomeric membranes, composed of over 50,000 microvillae, each only a few tens of nanometers in diameter (see Suzuki et al., 1993). In this paper, we describe a genetic approach to this problem and demonstrate that the Drosophila light-activated conductance is composed of the TRP and TRPL channels. We studied in detail the physiology of *trp*, *trpl*, and *trpl; trp* double mutants and showed that TRP is the major, and TRPL a minor, component of the light-activated channels.

Do TRP and TRPL form multimeric channels? Our studies do not exclude the possibility that TRP and TRPL form multimeric channels, either together or in combination with additional subunits. However, our results demonstrate that TRP and TRPL are capable of responding to light-activation independently of each other, and that if additional subunits are involved, their function is fully dependent on the presence of TRP or TRPL.

How are the channels gated? Given that the vertebrate light-activated conductance is composed of cGMP-gated channels (Fesenko et al., 1985), much debate has arisen about the role of cGMP as an intracellular messenger in invertebrate phototransduction. Although there is no evidence of light-induced changes in cGMP levels in Drosophila, a number of models invoking a biochemical link between IP_3-dependent calcium increases and activation of a guanylate cyclase have been proposed (Bacigalupo et al., 1990). Recent studies relying on the application of membrane-permeable cGMP analogs (Bacigalupo et al., 1995) and the isolation of a cGMP-gated channel expressed in sensory neurons (Baumann et al., 1994), including photoreceptor cells, have been used as support for this model. Our finding that the light-activated conductance is dependent on TRP and TRPL, neither of which is a cyclic nucleotide–gated channel, does not support the idea that cGMP is involved in gating the light-activated channels. Instead, evidence from heterologous expression studies suggesting that TRP is gated by depletion of the internal stores (Vaca et al., 1994) point toward a gating mechanism resembling I_{crac} (Hoth and Penner, 1992). We show that TRP and TRPL are coexpressed in the rhabdomeres, strongly suggesting that neither TRP nor TRPL is gated by a store-operated calcium release mechanisms based on direct conformational coupling with a receptor on the SRC. The availability of *trp* and *trpl* mutants now allows for a detailed study of their gating mechanisms and for a comprehensive dissection of channel function and regulation in a well-defined PLC signaling pathway amenable to genetic manipulation.

Experimental Procedures

Mutant Screen and Western Blots

Males of *cn bw* genotype were aged for 2 days, treated with ethyl methanesulfonate, and crossed en mass to flies carrying the dominant temperature-sensitive DTS91 allele. Single F1 males were collected and crossed in single vials to CyO/DTS91 virgin females. The vials were then shifted to 29°C for 72 hr to eliminate any eggs or larvae carrying the DTS allele. The parents were then removed and the vials were incubated at 29°C for an additional 48 hr before returning to 25°C. The progeny from this cross were transferred to fresh food, and their homozygous white-eyed offspring (*cn bw*) were subjected to a protein immunoblot screen for the loss of the TRPL antigen (see Dolph et al., 1993, 1994). In essence, single fly heads were removed and sonicated for 3 s in SDS Laemmli buffer. Samples were loaded on a 10% SDS–polyacrylamide gel electrophoresis (1 head per lane); proteins were allowed to enter the gel for 10 min, and then protein extract from a second mutant was loaded to minimize the number of gels. On a single gel, 30 individual flies representing 30 treated chromosomes could be screened. Blots were incubated with the anti-TRPL antibody and a control antisera (anti-$dG_{q\alpha}$; Scott et al., 1995) to check for the amount of extract loaded in the gels. We used an antibody generated against a 15 amino acid peptide from the C-terminal end in all the screens (see below).

Antibodies

To generate antibodies specific to TRP and TRPL, we synthesized peptides corresponding to regions unique to each protein. These included residues 11–26 (KALGSRLDYDLMMAEE) of TRP and residues 1082–1097 (DNSNFDIHVVDLDEK) of TRPL. We also used a TRP polyclonal antibody directed against the last 300 amino acids of the protein (Montell and Rubin, 1989) with similar results. Peptides were coupled to carrier protein, injected into rabbits and rats and antisera was affinity purified as previously described (Cassill et al., 1991). All antibodies were checked for specificity and affinity using wild-type, mutant, and transgenic controls. For immunostaining, the TRPL antibody was diluted 1:100 in phosphate-buffered saline (PBS), 1% bovine serum albumin, 0.1% Saponin (PBS-S); the TRP antibody was first preabsorbed with a homogenate of ~50 *trp* mutant heads to reduce background staining and used at a final dilution of 1:300. Rhodopsin and PLC were localized using an anti-RH1 monoclonal antibody (de Couet and Tanimura, 1987; 1:300 dilution) and a rabbit polyclonal anti-PLC antibody (1:1000 dilution), respectively.

Immunocytochemistry

Fly heads were cut and fixed in 3% paraformaldehyde, 5 mM EDTA in PBS for 1 hr on ice and infiltrated overnight with 2.3 M sucrose in PBS at 4°C. Heads were then cut in half, mounted, and quick frozen in liquid nitrogen. Sections of 1 μm were cut on an ultramicrotome at −80°C and collected on gelatin-covered slides (Smith et al., 1991). After blocking for 1 hr at room temperature in PBS-S, slides were incubated with primary antibody in PBS-S either for 4 hr at room temperature or overnight at 4°C. Samples were incubated with secondary fluorescent antibodies for 1 hr at room temperature. After washing, samples were mounted in glycerol with an antibleaching agent, p-phenyldiamine (1 mg/ml in 9 parts glycerol, 1 part 1 M Tris–HCL [pH 8.5]). Samples were digitized using a cooled charge-coupled device camera (Spectrasource) attached to a Nikon optiphot-2 microscope and processed using NIH Image and Adobe Photoshop software. Immunoelectron microscopy, fixation, and detection procedures were exactly as previously described (Suzuki et al., 1993; Suzuki and Hirosawa, 1994); anti-TRP and anti-TRPL antibodies were detected using secondary antibodies coupled to 5 or 10 nm colloidal gold.

PCR Reactions

The *trpl* genomic region from wild-type and $trpl^{802}$ mutant flies was amplified as two overlapping fragments in independent PCR reactions by the polymerase chain reaction (Smith et al., 1991). We also amplified first-strand cDNA from the mutant flies (RT–PCR) to confirm the defect at the mRNA level. PCR products from multiple PCR reactions were sequenced in each case to eliminate possible errors occurring during PCR amplification. PolyA-containing RNA was isolated from mutant heads and wild-type controls as previously described (Zuker et al., 1985). PCR reactions were carried out using Taq polymerase.

Electrophysiological Recordings

Photoreceptors were isolated from adult flies (<6 hr after eclosion) and whole-cell, patch-clamp recordings were performed as previously described (Ranganathan et al., 1991). Photoreceptors were stimulated by a 75 W Xenon source connected to the epifluorescence port of an inverted microscope; light was band-pass filtered ($\lambda = 580 \pm 10$ nm) and focused onto the photoreceptor cells with a 0.5 numerical aperture, 40× objective. Unattenuated output from

the objective at 580 nm was 0.09 mW. Signals were recorded with an Axopatch 1-D patch-clamp amplifier (Axon Instruments, Foster City, CA) and data were analyzed using pClamp5.51 and pClamp6 software (Axon). The membrane potential of the photoreceptors was voltage-clamped at a holding potential of −60 mV, and ~80% series resistances were compensated. Unless stated otherwise, the bath solution contained 120 mM NaCl, 5 mM KCl, 10 mM HEPES, 5 mM Proline, 29.5 mM Sucrose, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$ (pH 7.15). The pipette solution contained 120 mM CsCl, 15 mM TEA–Cl, 10 mM HEPES, 0.1 mM $MgCl_2$, 0.1 mM EGTA, and 3 mM Mg^{2+}-ATP (pH 7.15). When lanthanum was used, magnesium and calcium were omitted from the external solutions; 10 μM lanthanum was either included from the beginning or was applied during the experiment. The internal solution in these experiments contained 140 mM K^+ gluconate, 10 mM HEPES, 2 mM $MgSO_4$, 3 mM Mg^{2+}-ATP (pH 7.15). Potentials were not corrected for a ~3 mV junction potential.

For quantum-bump analysis, photoreceptors were clamped at −80 mV, and stimulated with dim light to generate quantum bumps <50% of the time (Scott et al., 1995). Signals were low-pass filtered at 500 Hz and sampled at 1 KHz. The pipette solution in these experiments contained 95 mM K^+ gluconate, 40 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$, 0.2 mM EGTA (pH 7.15); the external solution contained 1.5 mM $CaCl_2$, but no magnesium. Experiments described in Figures 5 and 6 used a light source with an unattenuated output of 0.04 mW at 580 nm.

DNA Constructs and Transgenic Flies

A 3500 bp *trpl* DNA fragment containing the entire coding region (Phillips et al., 1992) was cloned into a Drosophila transformation vector under the control of the heat-shock promoter (Baker et al., 1994), and injected into wild-type and *trpl* mutant embryos. P element–mediated germline transformations and all subsequent fly manipulations were performed using standard techniques. All experiments involving *trp* used the trp^{301} allele.

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