Molecular Neurobiology

ISSN0893-7648/96/12(2): 163–180/$9.50

Role of *Drosophila* TRP in Inositide-Mediated Ca^{2+} Entry

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Abstract

Inositol lipid signaling relies on an $InsP_3$–induced Ca^{2+} release from intracellular stores and on extracellular Ca^{2+} entry, which takes place when the Ca^{2+} stores become depleted of Ca^{2+}. This interplay between Ca^{2+} release and Ca^{2+} entry has been termed capacitative Ca^{2+} entry and the inward current calcium release activated current (CRAC) to indicate gating of Ca^{2+} entry by Ca^{2+}-store depletion. The signaling pathway and the gating mechanism of capacitative Ca^{2+} entry, however, are largely unknown and the molecular participants in this process have not been identified. In this article we review genetic, molecular, and functional studies of wild-type and mutant *Drosophila* photoreceptors, suggesting that the *transient receptor potential* mutant *(trp)* is the first putative capacitative Ca^{2+} entry mutant. Furthermore, several lines of evidence suggest that the *trp* gene product TRP is a candidate subunit of the plasma membrane channel that is activated by Ca^{2+} store depletion.

Index Entries: Ca^{2+} mobilization; genetic dissection; phototransduction; inositol lipid signaling.

Introduction

Inositol lipid signaling results in an elevation of cellular Ca^{2+}, which is derived from two different sources; intracellular and extracellular. This dual process of Ca^{2+} mobilization is controlled by two different information flow pathways operating in opposite directions. One pathway is initiated at the cell surface on activation of a specific receptor, leading to G-protein-mediated activation of phospholipase C and generation of inositol trisphosphate ($InsP_3$) *(1)*. It culminates in the release of Ca^{2+} from internal stores on binding of $InsP_3$ to an $InsP_3$-receptor that resides on the membrane of the internal Ca^{2+} stores *(2–4)*. The second pathway is initiated at the cell interior and takes place on decrease in the content of Ca^{2+} in the stores. The information on the state of filling of Ca^{2+} in the stores is relayed back to the plasma membrane, resulting in Ca^{2+} influx from the medium into the cell, thereby replenishing Ca^{2+}

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in the internal stores *(5–11)*. Although the two pathways are interconnected, each can be demonstrated separately under specific experimental conditions:

1. Selective release of Ca^{2+} from internal stores is demonstrated when cells are bathed in a Ca^{2+}-free medium containing Ca^{2+}-chelator. Under these conditions activation of the receptor gives rise to transient increase in cellular Ca^{2+} that is extinguished as soon as the internal stores are depleted of Ca^{2+}.
2. To demonstrate influx of Ca^{2+} from the external medium, the internal Ca^{2+} stores are depleted of Ca^{2+} either by activation of the membrane receptor in cells incubated in Ca^{2+}-free medium or by application of specific inhibitor of the Ca^{2+} uptake into the store like thapsigargin or cyclopiazonic acid. In spite of a constant leak of Ca^{2+} from the internal stores, the Ca^{2+} stores are not depleted because of efficient Ca^{2+} pumping by powerful Ca^{2+} ATPase. Depletion of the stores can therefore be achieved by inhibition of Ca^{2+}-store pumping with thapsigargin.

These manipulations change the permeability of the cell membrane to Ca^{2+}, as can be easily demonstrated on readdition of Ca^{2+} to the medium. Under these conditions Ca^{2+} influx is detected even when the receptor is blocked by antagonist and it lasts until the internal Ca^{2+} stores are refilled. Drawing an analogy to a capacitor in an electrical circuit, fully charged Ca^{2+} stores prevent Ca^{2+} influx whereas Ca^{2+} entry is promoted when the stores are depleted of Ca^{2+}. This hypothetical model of Ca^{2+} entry regulation was therefore termed capacitative Ca^{2+} entry and its essential features can be demonstrated in a great variety of cells, such as T-lymphocytes, fibroblasts, osteoclasts, mast cells, *Xenopus* oocytes, and other vertebrate cells (reviewed in refs. *10–12*). The common properties that characterize capacitative Ca^{2+} entry in various vertebrate cells are:

1. Activation by agonists or pharmacological agents (in the presence of strong Ca^{2+} buffers), that release Ca^{2+} from the internal stores, including $InsP_3$, Ca^{2+}-ionophores, and thapsigargin.
2. Very low unitary chord conductance and high selectivity for Ca^{2+} *(12,13;* but *see* ref. *14)*.
3. Ca^{2+} entry efficiently blocked by lanthanum (La^{3+}).
4. Ca^{2+} entry mechanism strongly affected in a dual manner by the cellular Ca^{2+} level: Low Ca^{2+} facilitates whereas high Ca^{2+} inactivates Ca^{2+} entry in complex and characteristic kinetics *(15,16;* for a review *see* ref. *10)*.

It should be pointed out that although the first pathway is well documented, its molecular components have been cloned and its individual reactions have been reconstituted using purified components, little is known about the operation of the second pathway of capacitative Ca^{2+} entry in vertebrate cells. Despite considerable efforts the components of the second pathway have not been identified and its mechanism of action remains largely unknown and highly controversial.

A promising approach to study these important but extremely complex signaling pathways is to genetically dissect the system using specific *Drosophila* mutants *(17–24)*. Phototransduction in *Drosophila* photoreceptors is mediated by inositol lipid signaling and many mutants defective in inositol lipid signaling have been isolated, cloned, and characterized by functional assays *(25–31)*. Several reviews on capacitative Ca^{2+} entry *(10–12)* and on *Drosophila* phototransduction *(19,23,24)* have been recently published. To avoid repetition of these excellent and detailed reviews, the present article concentrates on a crucial component of the *Drosophila* phosphoinositide-mediated Ca^{2+} entry pathway—the transient receptor potential mutant *trp* and its gene product, the TRP protein *(32,33)*. This protein constitutes an essential component of the main route of Ca^{2+} entry *(21,23,34–36)*. We will describe genetic, molecular, and functional studies of the *trp* mutant and discuss implications imposed by these studies on the current models of capacitative Ca^{2+} entry in vertebrate cells.

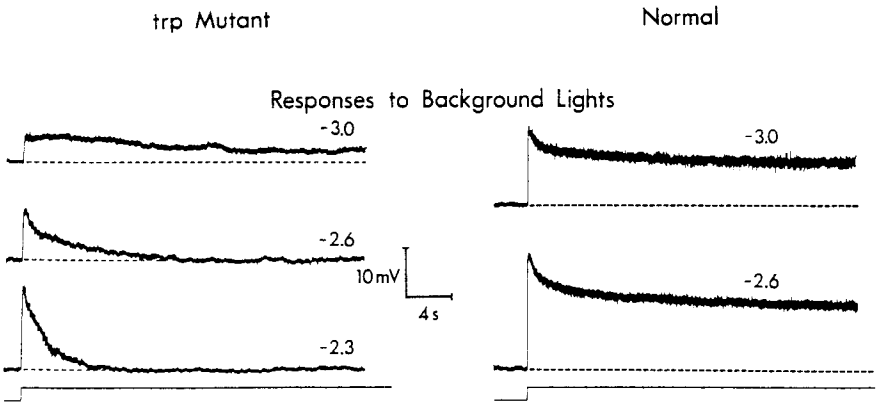


Fig. 1. A comparison between the intracellularly recorded receptor potentials of *trp* mutant and normal *Drosophila* photoreceptors to stimuli of different intensities. Green (521 nm) lights of variable intensities (indicated in relative log scale) lead to a reduction in the receptor potential amplitude to baseline in *trp*. This reduction in amplitude is accompanied by a decrease in noise level in the mutant fly. No decrease in the plateau was observed in the normal fly. The bottom trace is the light monitor (modified from ref. *39*).

The TRP Protein Is Required for Sustained Excitation

The trp *Mutation Leads to a Reduction in Excitation Efficiency During Illumination*

The transient receptor potential (*trp*) mutant is a third chromosome recessive *Drosophila* mutant produced spontaneously *(37,38)*. In response to a short (<1 s) or a dim light, the receptor potential of the *trp* mutant is similar to that of wild-type. However, the responses to illumination with light steps (>5 s) of increasing intensities decline exponentially to baseline, with a rate that accelerates on an increase of the stimulus intensity within a narrow range of light intensities (Fig. 1). After complete response inactivation, when the photoreceptor no longer responds to light, the sensitivity to light recovers within 1 min in the dark *(39)*.

Several lines of evidence indicate that the decline of the light response in *trp* is caused by exhaustion of excitation. Consequently, during illumination the response to continuous intense light becomes equivalent to a response to dim light and then to darkness *(38,39)*. Shot noise analysis and observations of the response to continuous light have shown that in wild-type *Drosophila*, the production of single photon responses (quantum bumps) increases linearly with increasing light intensity *(40,41)*. In contrast, in the *trp* mutant bump production decreases on increasing the light intensity *(38)*. This is best illustrated in the putative *trp* homolog of the larger fly *Lucilia*, in which the quantum bumps are much larger than in *Drosophila*. In the no steady state (*nss*) mutant of *Lucilia (42)* the decrease in bump production during continuous illumination is readily observed. When the response of the *nss* mutant to intense light approaches baseline the quantum bumps almost disappear (Fig. 2B, upper trace), unlike the response to dim light, which shows steady production of quantum bumps during illumination (Fig. 2B, lower trace; *43*). Furthermore, in wild-type *Drosophila* background lights decrease the latency of the response to a test flash, whereas in *trp* background lights increase the latency of the response to light *(39)*. In invertebrate photoreceptors the duration of the latency strongly depends on the intensity of the light stimulus becoming several tens of milliseconds in response to dim lights *(44)*. An increased latency and a slower rise time of the response are indicative of a response to dim lights in invertebrates. In *trp* such responses are observed on illumination with intense light

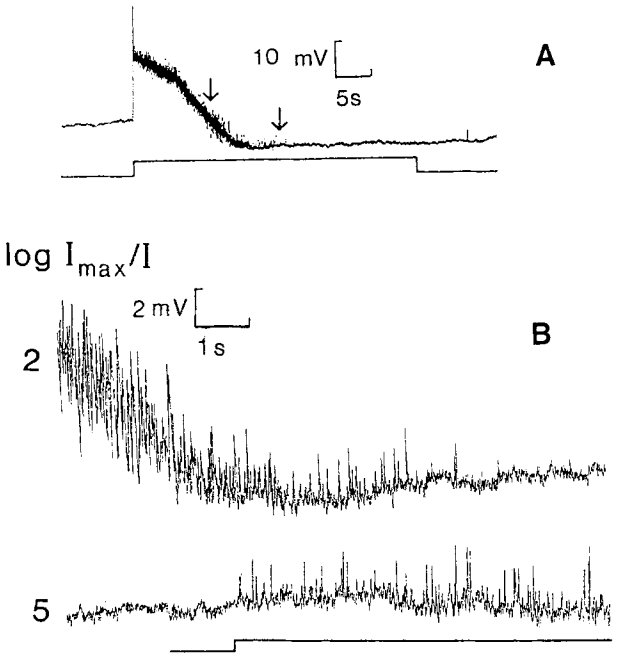


Fig. 2. The decline of the receptor potential of the *nss* mutant is accompanied by a reduction in the rate of occurrence of the quantum bumps, with little or no change in their amplitude and shape. **(A)** A receptor potential of the *nss* mutant in response to orange (OG–590) light with maximal intensity attenuated by 2 log units (log $I_{max}/I = 2.0$). **(B)** Upper trace: a magnified time region of the response of (A) between the arrows. A continuous reduction in bump production up to complete disappearance of the bumps is demonstrated by a continuous decrease in noise level. Lower trace: a response recorded from the same cell but in response to dim orange light with maximal intensity attenuated by 5 log units (log $I_{max}/I = 5.0$) showing bump production (noise level) at steady state level (modified from ref. *43*).

superimposed on background illumination, which partially exhausts excitation (Fig. 3).

Diminished Cellular Ca^{2+} Underlies the *trp* Phenotype

The *trp* phenotype can be explained by the hypothesis that a critical component in a specific cellular compartment of the photoreceptor cell is required for excitation. This component, which is used up during excitation, needs continuous replenishment in order to maintain a sustained response. In the *trp* mutant this critical component is exhausted because it cannot be replenished fast enough because of mutation in the *trp* gene. The identification of this critical component should help to unravel the mechanism that underlies the *trp* phenotype. The identification is based on an artificial depletion of the compound in wild-type, thereby conferring on wild-type a *trp* phenotype. Reciprocally, exogenous application of this substance to the *trp* mutant should rescue the mutant phenotype.

Application of La^{3+} Confers on Wild-Type a trp *Phenotype*

Application of lanthanum (La^{3+}) to the extracellular space of several species of wild-type flies accurately mimic the *trp* (or *nss*) phenotype (*45,46;* Fig. 4). Shot noise analysis indicates that the rate of occurrence of the single photon responses (quantum pumps) decreases in La^{3+}-treated wild-type flies in parallel with the increase in the intensity of illumination within a narrow range of light intensities similar to the effect of light on the mutant. Furthermore, application of La^{3+} to the mutant has virtually no effect *(46)*. The effect of La^{3+} is reversible on removal of La^{3+} from the external solution. Since La^{3+} is known as Ca^{2+}-channel blocker, it was suggested that the exhaustion of excitation in La^{3+}-treated wild-type flies or in the *trp* mutant is caused by inhibition of the mechanism responsible for replenishing cellular Ca^{2+} in a specific compartment, which must take place during strong illumination, and that some level of cellular Ca^{2+} in this specific compartment is required for excitation *(21)*.

A much stronger effect of La^{3+} on the light response was found in another invertebrate, the barnacle. In the barnacle external La^{3+} completely abolishes the response to light whereas iontophoretic application of Ca^{2+} into the cell in the presence of La^{3+} rescues a transient response to light *(47)*. Interestingly, application of La^{3+} to *Limulus* ventral photoreceptors had virtually no effects. It has been suggested that

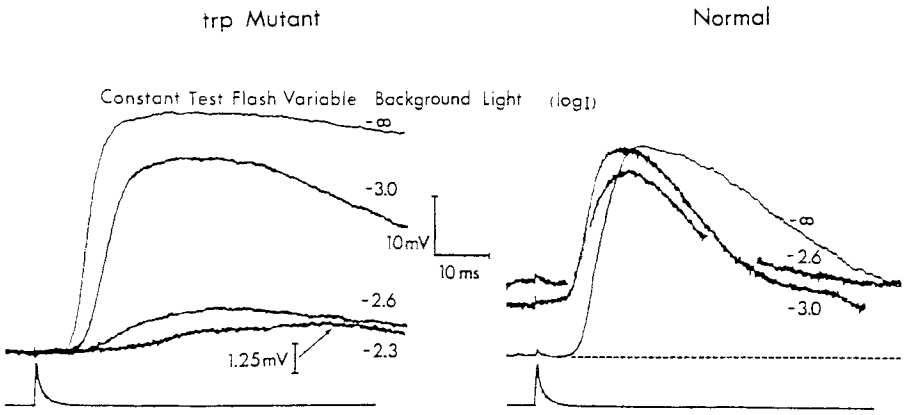


Fig. 3. A comparison between the intracellularly recorded receptor potentials of *trp* mutant and normal *Drosophila* photoreceptors to flash stimuli of constant intensity. In *trp* dim green (521 nm) background lights of variable intensities lead to a reduction in the receptor potential amplitude. This reduction in amplitude is accompanied by an increase in latency in the mutant fly, but by a decrease in latency in the normal fly (wild-type). In wild-type, the positive shift in baseline reflects the amplitude of the response to the background illumination. The constant test flashes (OG 515 edge filter) used were obtained by attenuating the light source having an intensity of 4.4×10^{15} photons/cm^2/flash (at 580 nm) by 1.0 and 3.0 log units for the mutant and normal fly, respectively. Tracings of the dark-adapted responses (-∞) of the same cells are also shown for comparison. The bottom traces give the light monitor (modified from ref. *39*).

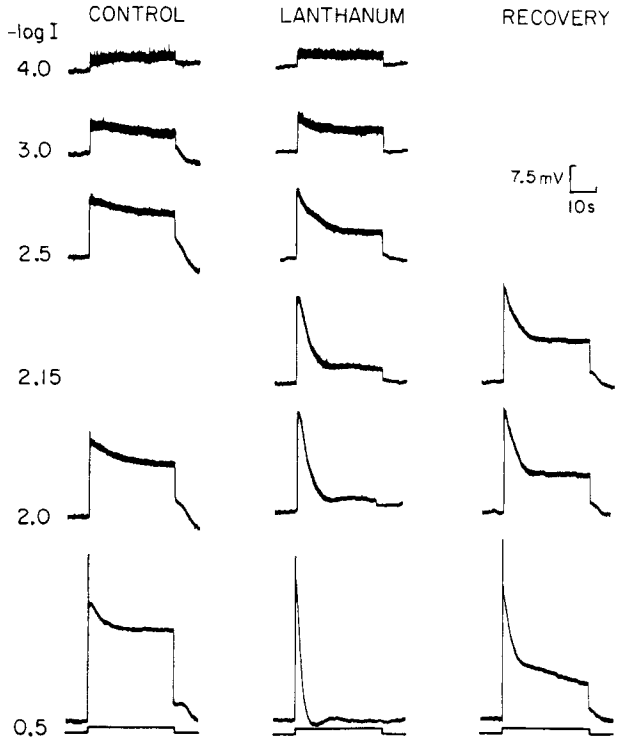


Fig. 4. The effects of lanthanum on the receptor potentials recorded from intact white-eyed *Musca* eye in response to increasing intensities of orange (Schott-OG 590 edge filter) lights as indicated. The left column shows control responses before application of La^{3+}; the middle column shows the responses of the same cell in the presence of La^{3+}. The right column shows partial recovery of the responses 20 min after the injection of La^{3+} (modified from ref. *46*).

the different effect of La^{3+} on photoreceptors of different invertebrate species is casued by variability in the size of the $InsP_3$-sensitive Ca^{2+} stores. Accordingly, when these stores are large (*Limulus*, bee) Ca-store replenishment is not a limiting factor, whereas in the fly and barnacle, which have very small $InsP_3$-sensitive Ca stores, replenishment of Ca^{2+} is a limiting factor leading to exhaustion of excitation when this process cannot cope with depletion of the Ca^{2+} stores *(47)*.

Prolonged Ca^{2+} Deprivation Confers on Wild-Type a trp *Phenotype*

A more direct correlation between the *trp* phenotype and exhaustion of Ca^{2+} stores was provided by exposure of isolated *Drosophila* ommatidia to Ca^{2+}-free medium for an extended

period (>30 min) while keeping cellular Ca^{2+} buffered with EGTA to ~50 n*M* Ca^{2+}_i. A typical *trp* phenotype was observed under such conditions *(34)*. Similar apparent deprivation of cellular Ca^{2+} could be obtained in the isolated ommatidia of wild-type *Drosophila* during a critical period of pupa development. At this critical developmental stage (P14) no response to light can be observed unless micromolars of Ca^{2+} are provided by the whole-cell recording pipet. Interestingly, the light response under such conditions has the typical characteristics of the *trp* phenotype *(48)*. Presumably under such conditions cellular Ca^{2+} is the limiting factor of excitation like in La^{3+}-treated or in Ca^{2+}-deprived wild-type cells.

The above experiments strongly suggest that the *trp* phenotype is related to exhaustion of Ca^{2+} in some cellular compartment caused by prolonged intense illumination. We have suggested that this cellular compartment is the submicrovillar cisternae (SMC) *(49)*, the specialized extensions of the smooth endoplasmic reticulum in juxtaposition to the surface membrane at the base of the microvilli *(21)*. The reverse experiment, namely the rescue of the *trp* phenotype in the *trp* mutant by Ca^{2+}, was not achieved when Ca^{2+} was applied to the extracellular space or injected directly into the cytosol. This latter observation suggests that if the *trp* phenotype is caused by exhaustion of cellular Ca^{2+}, it must be depleted from some inaccessible compartment that can be refilled only via the TRP protein. Accordingly, if the TRP protein is missing (in the *trp* mutant) or its action is blocked (by La^{3+}) or being temporarily inefficient (in the pupae), Ca^{2+} application cannot rescue the *trp* phenotype.

TRP Is Required for Light Adaptation

In addition to its critical role in maintaining sustained excitation, TRP also plays a critical role in mediating the gain control and the kinetic properties of the light response. These properties are collectively known as light adaptation. The mechanism of light adaptation enables the photoreceptors to operate efficiently over a wide range of ambient light intensities without reaching saturation. Light adaptation is known to be mediated via an increase in cellular Ca^{2+} *(50)* and is best illustrated by a shift of the intensity-response function (V-log I curve) to higher levels of light intensities. In the *trp* mutant, there is virtually no shift of the V-log I curve although further increase in the intensity of background illumination brings about response compression, i.e., the V-log I curve is reduced by a scaling factor (Fig. 5). The mechanism underlying light adaptation has been extensively studied in *Limulus* ventral photoreceptors *(51)*. It has been shown that a release of Ca^{2+} from the SMC by light or by $InsP_3$ feeds back in a positive manner to facilitate excitation and then in a negative manner to inhibit excitation and further Ca^{2+} release *(52,53)*. This dual action of Ca^{2+}, producing both positive and negative feedback effect on the $InsP_3$-sensitive Ca^{2+} stores, has been found in many $InsP_3$ systems *(1,10,16)*. In the *Limulus* photoreceptors, the negative feedback of Ca^{2+} on excitation seems to mediate light adaptation via inhibition of Ca^{2+} release from internal stores *(53–55)*.

In *Drosophila* photoreceptors, the positive and negative feedback of Ca^{2+} on excitation is mediated by Ca^{2+} entry *(56,57)* via a TRP-dependent mechanism *(58,59)*. Accordingly, the positive and negative feedback, typical of wild-type photoreceptors, are absent in the *trp* mutant. The lack of feedback regulation of the light-induced current (LIC) in *trp* is probably responsible for the lack of light adaptation in this mutant. The absence of light adaptation in *trp* may simply arise indirectly as a secondary consequence of a reduced Ca^{2+} influx into the cell or alternatively, it may be caused by a specific property of TRP that mediates the feedback control. Another possibility is that TRP mediates a local increase of Ca^{2+} in a critical restricted area and that only TRP can elicit such local increase in Ca^{2+}.

Two main methods have been applied to measure Ca^{2+} influx into invertebrate photoreceptors: measurements of the light induced increase in cellular Ca^{2+} using optical methods

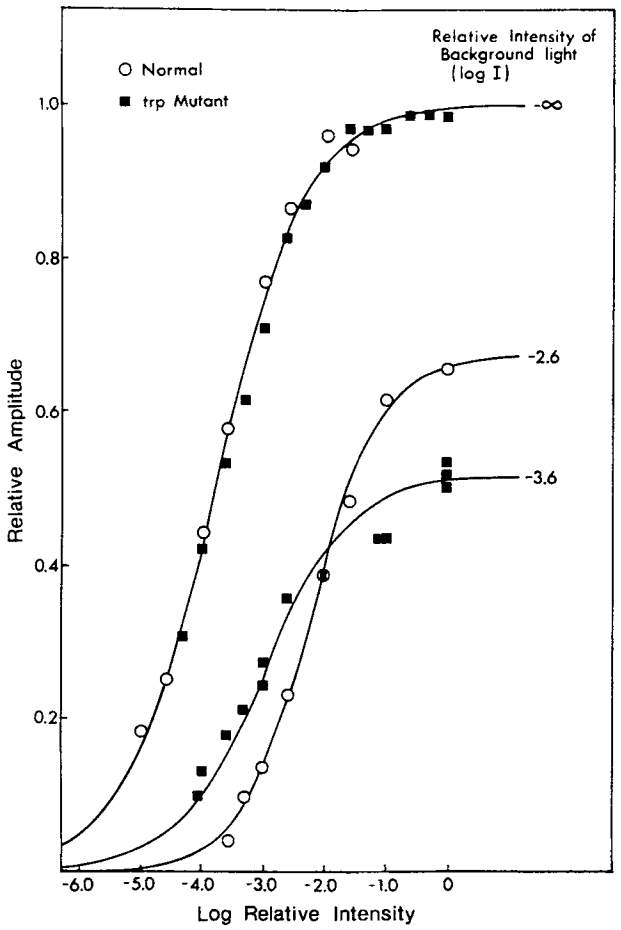


Fig. 5. Intensity-response functions measured intracellularly from a single normal (O) and a single *trp* photoreceptor (■). All the points were measured at the peak of the receptor potential in response to increasing intensities of 524-nm green light pulses. The upper curve was measured in dark-adapted cells and the other two curves were measured from the incremental responses during 521-nm background lights of various intensities as indicated. In normal *Drosophila* but not in *trp*, the responses are superimposed on the response to the background illumination. The smooth curves were calculated using the hyperbolic function ($v/v_{max} = I^n/(I^n + \sigma^n)$, $n = 0.6$ and $\sigma = 3.7$ for the upper curve, $n = 0.6$ and $\sigma = -3.0$ for the lower *trp* curve, and $n = 0.75$ and $\sigma = 0.75$ and $\sigma = -2.2$ for the light-adapted normal fly (from ref. *39*).

(bioluminescence of photoproteins or fluorescence of Ca-indicators), or light-induced decrease of extracellular Ca^{2+} using Ca^{2+}-selective microelectrodes.

TRP and Ca^{2+} Influx

In insects and barnacle photoreceptors, light-activates a substantial Ca^{2+} influx that persists during sustained illumination. This light-activated Ca^{2+} influx was demonstrated in the barnacle lateral ocellus using bioluminescence of the Ca^{2+}-sensitive protein, aequorin *(54)*, and in the honey bee drone using Ca-indicator fluorescence *(60)* and Ca^{2+}-selective microelectrodes *(61,62)*. Whole-cell voltage clamp recordings from isolated *Drosophila* ommatidia have shown that the light-activated channels are ~40 times more permeable to Ca^{2+} than to Na^+ *(56,57)*. The critical role of TRP in producing high Ca^{2+} permeability of the light-activated channels was demonstrated by Hardie and Minke *(34)*. Measuring the reversal potential (E_{rev}) of the LIC as a function of external Ca^{2+} concentration showed that the high Ca^{2+} permeability of the light-activated conductance is reduced ~10-fold in the *trp* mutant *(34)*. A reduced light-activated Ca^{2+} influx in *trp* and *nss* was found by direct measurements of the decrease in external Ca^{2+} concentration during illumination. Measurements in the intact fly showed that the light-induced Ca^{2+} influx was markedly reduced in the *trp* and *nss* mutants as compared with Ca^{2+} influx of wild-type flies *(35,63)*. Similar results were obtained by simultaneous measurements of LIC and change in intracellular Ca^{2+} using Ca-indicator fluorescence in isolated ommatidia of wild-type *(36,64)* and the *trp* mutant (*36;* Fig. 6).

Is the decreased Ca^{2+} permeability of the light-activated channels in the *trp* mutant the primary cause of the exhaustion of excitation? Are there other properties of TRP that are essential for sustained excitation? These critical questions cannot be satisfactorily answered at our present state of knowledge. Nevertheless, the recent measurements of light-induced Ca^{2+} influx in the *trp* mutant in vivo indicate that during the transient light response significant amounts of Ca^{2+} enter the cell. Therefore, the reduced Ca^{2+} permeability of *trp* is not sufficient to explain the *trp* phenotype.

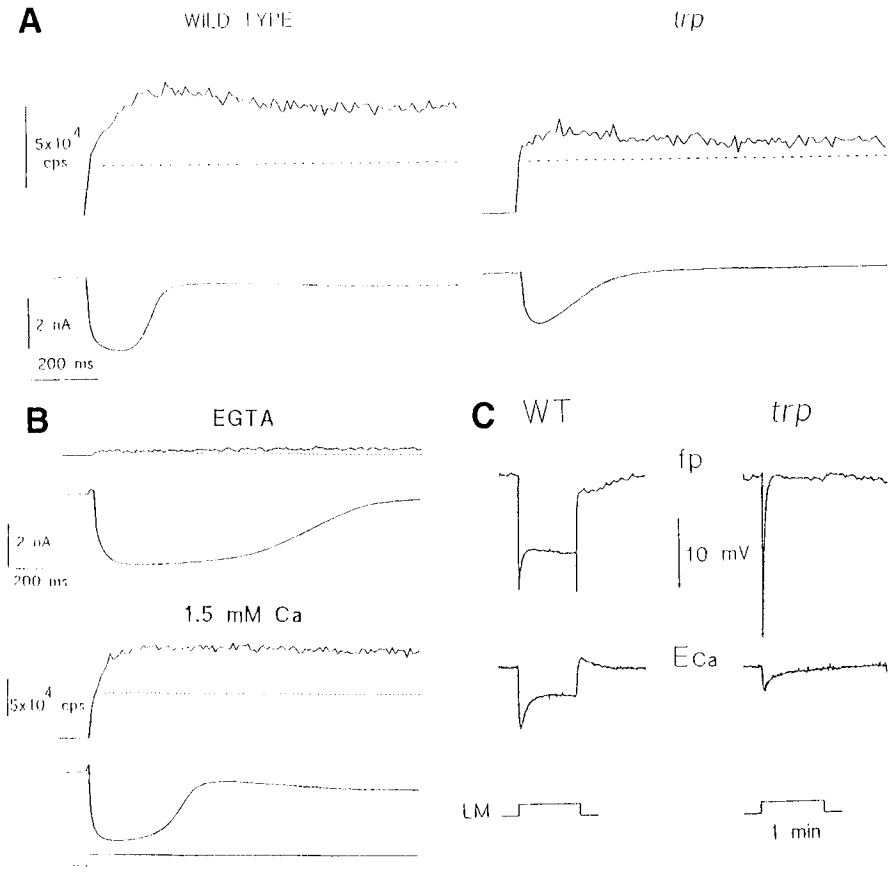


Fig. 6. Light-induced Ca^{2+} influx in *Drosophila* photoreceptors is blocked by removing external Ca^{2+} and reduced by the *trp* mutation. **(A)** Calcium Green–5N (100 μ*M*) fluorescence (upper) and LIC (bottom) measured simultaneously in adult (< 4 h) wild-type *Drosophila* photoreceptors (left) and in the *trp* mutant (right) at –50 mV holding potential. The *trp* mutation largely (about threefold in average) reduced the Ca^{2+} signal relative to wild-type. The dotted line indicates the resting Ca^{2+} level in the photoreceptor. **(B)** Simultaneous measurements of fluo-3 fluorescence (500 μ*M*, upper trace) and LIC (lower trace) in wild-type photoreceptors voltage clamped at –50 mV, 6 min after Ringer's solution containing no added Ca^{2+} and 0.2 m*M* EGTA was applied. The bottom traces show similar measurements obtained 3 min after 1.5 m*M* Ca^{2+} was added to the Ringer's solution. **(C)** Light-induced Ca^{2+} changes in the retinal extracellular space of wild-type (left) and *trp* mutant of *Drosophila* (right) recorded from intact eyes under identical conditions using Ca-selective microelectrodes. The top traces are the extracellularly recorded receptor potentials (field potential: *fp*). The middle row is the light-induced potentiometric potential changes (ΔE_{Ca}) of the calcium-selective barrel of the Ca^{2+} microelectrode. The bottom traces give the time scale and the light monitor (LM). The figure shows that ΔE_{Ca} is largely reduced in *trp* relative to wild-type, indicating reduced Ca^{2+} influx. (from ref. *23*; modified from refs. *35,36*).

TRP Has a Structure of a Novel Ca^{2+} Channel Subunit

The trp *and* trpl *Genes*

The *trp* gene was cloned and sequenced by Montell and Rubin (*32*) and by Wong et al. (*33*). The *trp* gene encodes a novel 1275 amino acid (aa) protein with six putative transmembrane domains. Flanking this hydrophobic region are *N*- and C-terminal domains of approx 300 and 600 residues. The last 250 aa are very hydrophilic and contain the following eight aa sequences repeated perfectly in tandem nine times: Asp-Lys-Asp-Lys-Lys-Pro-Gly/Ala-Asp.

In a search for genes that encode calmodulin-binding proteins, Kelly and colleagues *(65)* isolated a new gene on the second chromosome of *Drosophila* with a considerable (~40%) identity to *trp*, which they called *trp*-like *(trpl)*. The amino acid identity between *trp* and *trpl* is not uniformly distributed throughout the sequences. There is considerable (56%) amino acid identity over the amino-proximal sequence mainly in the membrane-spanning domain (residues 50–340). The central region consists of two segments: the amino-terminal segment (residues 340–500), with 29% identity, and the remainder (residues 500–700), which shows 74% identity. The level of identity falls off dramatically (to 17%) over the carboxy-terminal regions of the proteins *(65)*.

Both TRP and TRPL have weak but significant homologies to known channel genes in the putative membrane-spanning region. The closest homology is with vertebrate voltage-gated Ca^{2+} channel α subunit (the dihydropyridine receptor). Unlike the α subunit of a voltage-gated Ca^{2+} channel, both TRP and TRPL contain only one of the four repeated channel units with six putative transmembrane domains known as S1–S6 *(65)*. By analogy to the voltage-gated K^+ channel and the cyclic nucleotide-gated channels, TRP may constitute one of the multiple subunits that form the channel. TRPL may constitute another subunit of the same channel or of another channel.

Special Features of TRP and TRPL

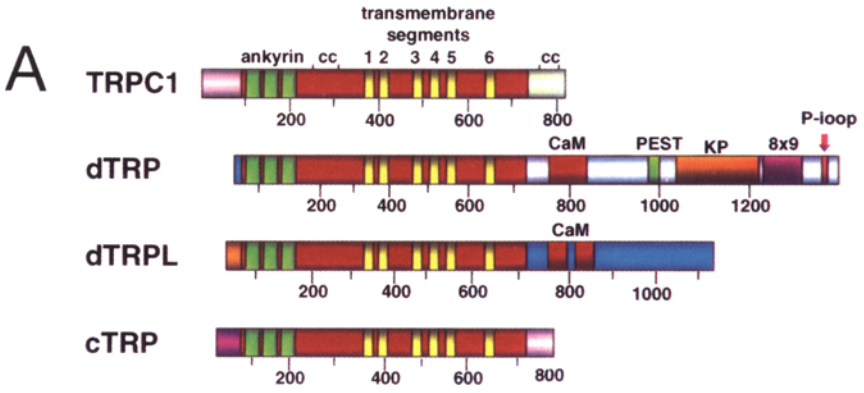
Calmodulin is known to bind to amino acid sequences that can form amphiphilic α helices with one face of the helix positively charged *(66)*. Two regions that show a high probability of forming an amphiphilic α helix were found in the *trpl*-encoded protein (residues 710–727 and 809–825, Fig. 7). When drawn in the form of an α helical wheel *(67)*, these sequences produce amphiphilic helices with a positively charged face and hydrophobic face, as expected of calmodulin-binding sites. A region homologous to the first putative calmodulin-binding site in the *trpl* protein is not present in the *trp* protein; however, the second site does show considerable homology (47% identity) with the equivalent region in the *trp* protein *(65)*. Although both TRP and TRPL have amphiphilic α helices sequence, only TRPL has been tested and shown to bind calmodulin *(65)*.

Residues 153–182 of the *trpl* protein and the equivalent region in the *trp* protein are comparable to the "ankrepeat" consensus sequence *(68)* having three ankyrin repeats *(69)*. Both sequences show considerable identity with individual members of the ankrepeats both from ankyrin *(68)* and from the *Drosophila Notch* protein *(70)*. Residues 929–960 of *trp* contain a PEST sequence, which is a signal for protein degradation by the calcium-dependent protease calpain, typical of calmodulin-binding proteins *(71)*. Immediately *N*-terminal to the 8 × 9 repeats there is a prolin-rich sequence in which the dipeptide KP is repeated 27 times. At the end of the C-terminus, immediately after residue 1256 there is a nucleotide-binding domain called the P-loop (Fig. 7).

TRP Homolog Genes

TRP homolog genes were identified in human cDNA fetal brain library (called TRPC1) and in *Caenorhabditis elegans* genome sequencing project (called cTRP) by Montell and colleagues *(69)*. Comparison of the deduced amino acid sequence with the gene and protein databanks demonstrated significant homology to the two *Drosophila* members of the TRP gene family. It was found that there is 38–40% identity and 58–62% sequence similarity between the human sequence and TRP, TRPL, and the *C. elegans* TRP over 625 amino acids (Fig. 7). In addition, there was a second region near the C-terminus that was approx 30% identical and 60% similar to each of the *trp* proteins. Overall, the human gene is as similar to the invertebrate *trp* genes as the invertebrate family members were to each other *(69)*.

The sequence identity between human TRP (TRPC1) and the invertebrate TRP proteins is significantly higher in the putative cytoplasmic regions and transmembrane segments (62%)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transmembrane segments
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1 2 3 4 5 6
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P-loop
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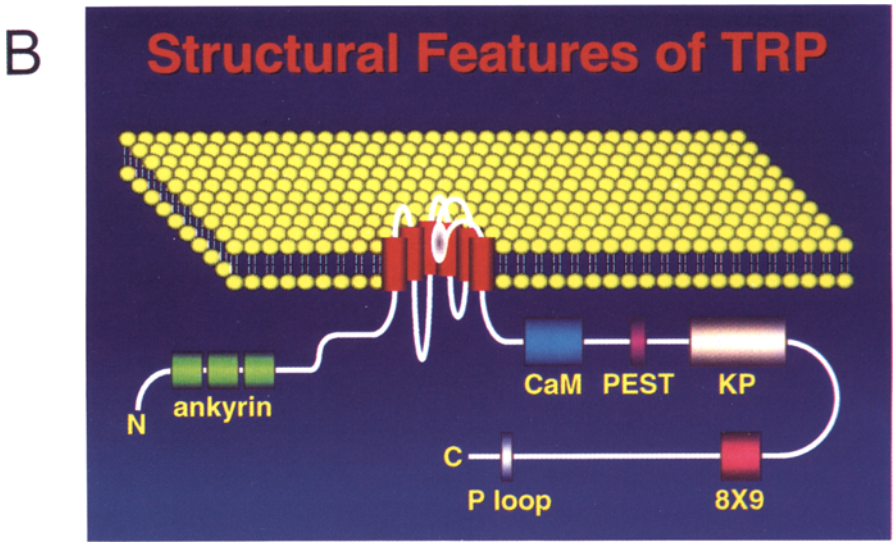
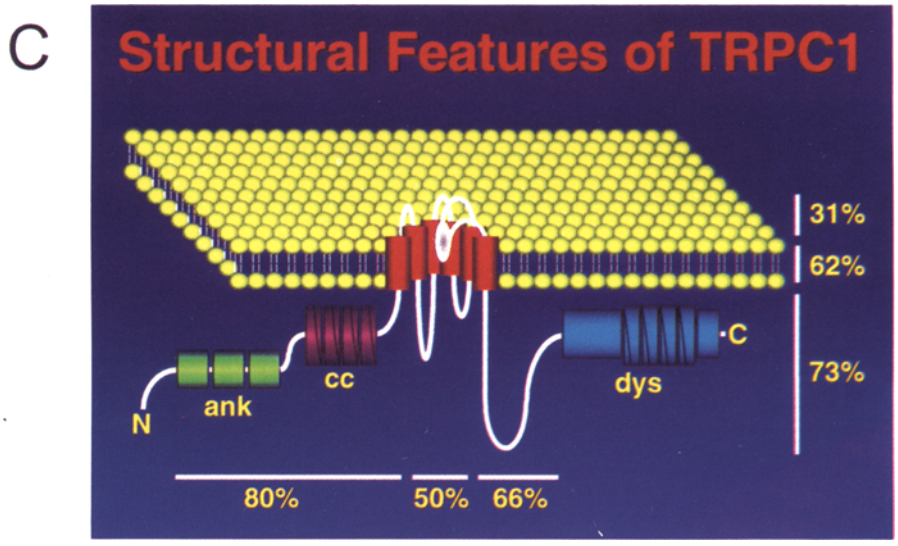

B
Structural Features of TRP
N
ankyrin
CaM
PEST
KP
C
P loop
8X9


C
Structural Features of TRPC1
31%
62%
73%
N
ank
cc
dys
C
80%
50%
66%

than in the putative extracellular loops connecting the putative transmembrane domains (31%, Fig. 7). The cytoplasmic region near the *N*-terminus of TRPC1 (residues 98–368) is 80% conserved and the domain C-terminal to the membrane-spanning segments is 66% identical (residues 664–720). The lowest conservation in the putative cytoplasmic region is in the two loops connecting the putative transmembrane segments (50%).

Like the N-terminal region of *Drosophila* TRP and TRPL, the *N*-terminal region of TRPC1 and cTRP also contained three ankyrin repeat motifs. Another domain, residues 256–300, is predicted to form a coiled-coil (cc) structure (84% probability) in TRPC1 and with low probability in TRPL and cTRP *(69)*. The C-terminal 90 amino acids of TRPC1, which had lower homology to the invertebrate TRP proteins, showed significant homology to dystrophin (dys). The *N*-terminal 95 amino acid sequence of TRPC1 is not homologous to any protein in the databanks. Like in *Drosophila* TRP and TRPL, in TRPC1 and cTRP the S4 segment does not have the positively charged residues that appear to constitute the voltage sensor of voltage-gated cation channels *(69,72)*.

Additional queries of cDNA database by Montell and colleagues *(69)* revealed two other putative members of the human TRP gene representing a second and third human member of the TRP family. In addition, protein sequencing of squid eye proteins revealed a polypeptide with marked identity to TRP (Findley, personal communication). Fragments from a *Xenopus* oocytes and mouse brain libraries include homologs of TRP *(73)*. All these recent findings indicate that the TRP represents a new multigene family of putative channel protein subunits found in many species along the evolutionary scale from the *C. elegans* to human. Since the ankyrin and the S1–S6 membrane regions have been conserved in all species, these regions seem to be the important functional regions of TRP. Therefore, the physiological studies in *Drosophila* are likely to shed light on the function of these channel subunit proteins in vertebrate species.

Fig. 7. *(previous page)* **(A)** Organization of *Drosophila*, TRP, and TRPL proteins (dTRP, dTRPL) in comparison to human (TRPC1) and *C. elegans* (cTRP) TRP homologs. All sequences contain six putative transmembrane helices S1–S6 (1–6), and a putative pore region between S5 and S6, S3–S6 show ~40% identity with the equivalent regions of the dihydropyridine receptor (a vertebrate voltage-gated Ca^{2+} channel). Both dTRP and dTRPL as well as TRPC1 and cTRP have three consensus ankyrin-binding motifs (ank) toward the amino terminus. This might represent a site for linkage to the cytoskeleton or interaction with another protein. There is one putative calmodulin-binding site (CaM) on the dTRP sequence, whereas dTRPL has two calmodulin-binding sites. These sites are missing in TRPC1 and cTRP. The TRP protein has a curious and unique hydrophilic sequence near the carboxyl terminus consisting of nine repeats of the sequence: DKDKKPG/AD (8 × 9). cc: coiled-coil domain; dTRP also has a PEST sequence, a proline-rich region with the dipeptide KP repeating 27 times and a P-loop sequence at the end of the C-terminus. **(B)** Putative domain structure and topology of dTRP. **(C)** Putative domain structure and topology of TRPC1. Percent identity between TRPC1 and at least one other member of the TRP family is indicated (dys: Dystrophin domain) (from ref. *69*). (Color photos courtesy of Craig Montell and Paul D. Wes.)

TRP Affects the Biophysical Properties of the Light-Activated Channel

The amino acid composition of TRP and TRPL, which have overall structure similar to voltage-gated channels, is a strong indication that TRP or TRPL are channel subunits. At present there is no sufficient data to determine if TRP or TRPL are the pore-forming subunits. Detailed analyses of the ionic selectivity of the light-activated channel of *Drosophila* are still lacking. The limited available data have been obtained by measurements of the reversal potential (E_{rev}) during ion-substitution experiments in whole-cell voltage-clamp recordings in isolated ommatida *(56,57)*. The light-acti-

vated conductance has a much higher permeability to divalent cations (Ca^{2+} and Mg^{2+}) than to monovalent ions ($P_{Ca}:P_{Mg}:P_{Na} = 40:8:1$). This conclusion is based on the dependence of E_{rev} on $[Ca^{2+}]_{out}$ and on a fit of the experimental data *(56)* to the constant field equation *(74)*. Interestingly, there is a similar low permeability of the light-activated channels to Na^+ and Cs^+, although both are less permeable than Mg^{2+} ($P_{Mg}:P_{Cs} = 8:1$, *56*). A large monovalent ion like $Tris^+$ has a permeability similar to Na^+ *(57)*.

The *trp* mutation or application of La^{3+} to the extracellular space have profound effects on the characteristics of the light-activated conductance:

1. The Ca^{2+} permeability of the light-activated conductance is reduced ~10-fold *(34)*.
2. Ca^{2+} influx is reduced about threefold *(35,36)*.
3. The *trp* mutation changes the voltage dependence of the light-activated conductance by eliminating a strong inward rectification of the conductance *(58,59,75)*. The latter effect may arise from reducing a partial Mg^{2+} block of the channel that was found in wild-type.
4. TRP is required for the Mg^{2+}-sensitivity of the channel.

In the absence of external Ca^{2+} about 95% of the LIC is blocked by physiological concentration (4 m*M*) of Mg^{2+} with $K_{1/2}$ of ~280 μ*M* Mg *(75)*. This Mg^{2+} block is relieved at both hyperpolarized (inward rectification) and depolarized (outward rectification) potential. In the *trp* mutant the Mg^{2+} block is greatly reduced (50% block $K_{1/2}$ 4 m*M* Mg^{2+}). The reduced Mg^{2+} block in *trp* may explain the large difference in single channel conductance between wild-type and *trp* obtained from shot noise analysis.

TRP Affects Single Channel Properties

The presumed location of the light-activated channels at the base of the microvilli *(76)* make the light-activated channels rather inaccessible to direct patch-clamp analysis. Moreover, fluctuation analysis (shot noise analysis) shows that the LIC is dominated by the noise arising from the quantum bumps. Therefore, at present the only available data on single channel activity in *Drosophila* photoreceptors has been derived from indirect measurements of the so-called run down current (RDC), which is a nonphysiological state of the photoreceptor. During prolonged whole-cell recordings the light-activated channels become spontaneously active because of a still unknown reason and are no longer under the control of the phototransduction machinery, resulting in a noisy inward current *(58)*. Shot noise analysis of wild-type RDC has provided effective single-channel conductance of ~3 ps (at –60 mV; 1.5 m*M* $Ca^{2+}{}_o$; 8 m*M* $Mg^{2+}{}_o$). This value may represent the average of two classes of channels. In the *trp* mutant or in La^{3+}-treated cells the calculated single channel conductance during RDC (at similar conditions) is about 10 times larger (~30 ps). This difference may represent the much lower sensitivity of the *trp* mutant to Mg^{2+} block. Under physiological divalent ion concentrations the spontaneous activation of the light-activated channels (RDC) appears in almost every photoreceptor of wild-type *Drosophila* under whole-cell recording but only rarely (1 out of 17 cells) in the *trp* mutant *(76)*, suggesting that the presence of TRP is required for RDC formation under normal divalent ion concentration.

Ca^{2+}-Dependent Inactivation of the Channels

During RDC, when the light-activated channels are constitutively open, an increase in cellular Ca^{2+} is obtained by stepping the holding voltage from positive to negative value, causing an increase in the driving force for Ca^{2+} entry. The increase in $Ca^{2+}{}_i$ blocks the RDC in a Ca^{2+}-dependent manner. This block is prevented in the presence of intracellular Ca^{2+} buffer, BAPTA, showing that Ca^{2+} blocks the channel from inside. This Ca^{2+} block is ~10 times faster than the negative feedback of Ca^{2+} on the LIC in responsive photoreceptors. Therefore, the Ca^{2+}-dependent inactivation seems to operate in a mechanism distinct from the negative feedback of Ca^{2+}, which mediates gain control at the quantum bump level *(58,59)*.

The profound effects of the *trp* mutation on the biophysical properties of the light-activated channels strongly support the hypothesis that TRP is a plasma membrane channel subunit. However, the available data is not sufficient to determine if TRP is part of the pore-forming subunit. Recent heterologous expression of TRP and TRPL do not help to solve this problem because of the limited similarity between the properties of the heterologously expressed conductance and the light-induced conductance of *Drosophila (77,78)*. Using Sf9 insect cell expression system and baculovirus-mediated infection, TRP and TRPL have been expressed individually in these cells. TRPL expression was manifested by the appearance of constitutive nonselective cation current *(77–79)*. This constitutive current could be enhanced by agonists of the G-protein-coupled receptors *(79,80)*. TRP expression was manifested by the appearance of cationic current on application of thapsigargin. The TRP or TRPL-dependent currents in the Sf9 cells markedly differ from the light-activated current of *Drosophila* in five main aspects:

1. In darkness the light-activated channels of *Drosophila* are not constitutively open.
2. The current-voltage relationship (I-V curve) of the LIC, which shows inward and outward rectification in *Drosophila,* is linear in Sf9 cells expressing TRP or TRPL.
3. There are no time-dependent kinetics of the expressed TRP or TRPL-dependent current unlike the LIC.
4. The TRP-dependent positive and negative feedback of Ca^{2+} on the LIC is completely absent in the Sf9 cells.
5. The TRP-dependent conductance in Sf9 cells is not blocked by Mg^{2+} in concentrations that have a strong blocking effect in *Drosophila*. Nevertheless, Sf9 cells or *Xenopus* oocytes expressing TRP reveal Ca^{2+} influx that is activated by Ca^{2+} store depletion with thapsigargin *(73,77)*.

Subcellular Localization of TRP

The subcellular localization of the TRP protein was determined using immunohistochemical staining with monoclonal anti-TRP antibody in newly emerged *Drosophila*. TRP was localized to the base of the microvilli in a region adjacent to the presumed $InsP_3$-sensitive Ca^{2+} stores (the SMC). This specific localization was supported by measuring the magnitude of a TRP-dependent inward current that results from spontaneous activation of the light sensitive channels during whole-cell recordings (RDC). It was found that reduction of the microvilli area through genetic dissection in the opsin null mutant, *ninaE^{ora} (19)*, was correlated with a pronounced enhancement of the TRP-dependent inward current relative to wild-type, suggesting that the TRP-dependent current was not produced along the length of the microvilli, at least in newly emerged flies. It was suggested that the functional localization of the TRP protein is on the plasma membrane loop found along the base of the rhabdomeric microvillus. Thus, the TRP channel may physically interact and functionally operate in concert with the $InsP_3$-sensitive Ca^{2+} stores *(76)*.

The Role of TRP in Capacitative Ca^{2+} Entry

The Role of TRP in Invertebrate Phototransduction

The phenotype of the *trp* mutant suggests that TRP must have a critical role in sustained excitation. Initially the possibility that TRP is a light-activated channel was ruled out because of the (almost) normal response of a *trp* mutant to dim or short lights *(32)*. To overcome this difficulty Hardie and Minke *(34)* have suggested that there are at least two light-activated channels: One channel is a nonselective cation channel (called the non-TRP channel), that is activated by light via $InsP_3$ production and Ca^{2+} release, giving rise to the light response in the *trp* mutant, whereas the other channel (the TRP-dependent channel) is a Ca^{2+} channel that gives the special properties of the light-activated conductance listed above *(34,72)*. The biphasic reversal potential of wild-type

observed at 0.5 m*M* external Ca^{2+}, the unique and more negative reversal potential of the LIC in the *trp* mutant, and the effects of La^{3+} support this hypothesis. An alternative hypothesis is that TRP is a subunit in heteromultimeric channel that gives all the special properties of the channel, including the Ca^{2+} permeability, the ability to refill the Ca^{2+} stores, the La^{3+} and Mg^{2+} sensitivity, and the ability to undergo run down. At the present state of our knowledge it is difficult to discriminate between these two alternatives. A critical but still unanswered question concerns the unknown property of TRP that is essential for sustained excitation. Clearly, this property is not a direct result of the reduced Ca^{2+} permeability because even in functionally null *trp* mutant intense light results in micromolar increase in cellular Ca^{2+} *(35)*.

TRP and the Capacitative Ca^{2+} Entry in Vertebrate Cells

Inositide-mediated Ca^{2+} entry has been implicated as a critical component in the function of a large variety of cells and tissues (for review, *see* refs. *1,5–8,10–12*). Unlike other voltage- and ligand-activated conductance, production of the second messenger $InsP_3$ does not seem to directly activate the surface membrane Ca^{2+} channel in photoreceptors *(81,82)*. Presumably, the activation of the surface membrane Ca^{2+} channels is indirect via release of Ca^{2+} from the $InsP_3$-sensitive Ca^{2+} stores. Also, the Ca-entry channel (I_{CRAC}) seems to have unusual characteristics, such as very low single-channel conductance (<3 ps), very high selectivity to Ca^{2+}, and unusual pharmacology (i.e., it is best blocked by La^{3+} and relatively less efficiently by Ni, whereas it is not blocked by organic Ca^{2+} channel blockers). The most controversial aspect of the capacitative Ca^{2+} entry is the gating mechanism of this channel. The dominant hypotheses are the production of still unidentified diffusible messages on Ca^{2+} store depletion, the so-called calcium influx factor (CIF) *(83)*, and the conformational coupling hypothesis, assuming protein–protein interaction between $InsP_3$ receptor and hypothetical plasma membrane Ca^{2+} channel that is gated by protein–protein interaction according to the state of filling of the Ca^{2+}-stores *(7–10)*.

TRP fulfills several of the properties required for the store-operated channel and provides an insight into a possible mechanism of the capacitative Ca^{2+} entry because:

1. TRP is necessary for inositide-mediated Ca^{2+} entry in *Drosophila* by a still unknown mechanism.
2. The current flow-through TRP-dependent channels can be activated by application of the Ca^{2+} inonophore, ionomycin, in the absence of external Ca^{2+} and in the presence of high concentration of internal Ca^{2+} buffer, BAPTA (Hardie, personal communication), just like the capacitative Ca^{2+} entry in mast cells *(12)*.
3. The TRP-dependent current shows strong inward rectification and is inactivated by internal Ca^{2+} like the capacitative current in vertebrate cells *(12)*.

However, at present there are several studies in *Drosophila* that do not support the hypothesis that TRP is activated by Ca^{2+} store depletion. These findings show that application of thapsigargin does not induce inward current in the dark *(64)* and that light-activated Ca^{2+} release from intracellular stores has not been demonstrated *(36,64)*. These results can be explained by the slow action of thapsigargin and the tiny dimensions of the SMC in *Drosophila*, resulting in detection difficulties. Clearly, more detailed and refined experiments are needed before conclusions can be derived from the above negative results.

The great advantage of TRP for investigating the capacitative Ca^{2+} entry mechanism is its known sequence. The fact that the "ank" repeat region and the transmembrane domain (S1–S6) are highly conserved from *C. elegans* to human indicates that these regions are critical for TRP function. The conservation of the transmembrane domain strongly suggests that TRP is a membrane channel subunit. The ankyrin repeat is known to be involved in protein–protein interactions and, most significantly, it is known to interact with the $InsP_3$ receptor *(3)*.

Perspectives

The *Drosophila* photoreceptor cells are the only system where inositol lipid signaling and its regulation can be analyzed by classical and molecular genetics. This approach has been recently complemented by electrophysiological analysis using whole-cell voltage clamp and biochemical studies of individual chemical reactions, which contribute functional assessment of the effect of mutations in particular genes.

Germ-line transformation of *trp* mutants using vectors with tissue-specific promoters and flies of null *trp* background as recipients should yield comprehensive information about the role of the *trp* domains in localization, function, and regulation of the TRP activity. This is also a suitable system to test the function of the human and *C. elegans trp* genes by testing if expression of human *trp* in *trp–Drosophila* rescue the *trp* phenotype.

Heterologous expression of TRP and TRPL is a powerful mean to study the function of these proteins in a well-controlled cellular environment. However, the Sf9-baculovirus expression system does not seem to be desirable because of the limited control on the level of protein expression that seems to be critical for TRPL. Other expression systems are required for efficient and well-controlled heterologous expression of TRP and TRPL.

Another source of information on Ca^{2+} mobilization in the *Drosophila* photoreceptor cells is a class of visual mutants that are called the *ina* PDA mutants *(17,19)*. Similar to *trp*, the *ina* mutants are also unable to maintain sustained excitation in response to continuous illumination with high intensity lights and undergo response inactivation. One mutant that belongs to this group is the *inaC* mutant, which has been found to be defective in a gene that encodes for an eye-specific protein kinase C *(28)*. Further analysis showed that *inaC* rapidly inactivates on removal of external Ca^{2+} and that the eye PKC is required for light adaptation *(30)*. If the other *ina* mutants are also defective in Ca^{2+} mobilization, analysis of the other *Drosophila ina* mutants will yield a comprehensive picture of Ca^{2+} homeostasis in the not too distant future.

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