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The Ordered Visual Transduction Complex of the Squid Photoreceptor Membrane

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Abstract

The study of visual transduction has given invaluable insight into the mechanisms of signal transduction by heptahelical receptors that act via guanine nucleotide binding proteins (G-proteins). However, the cyclic-GMP second messenger system seen in vertebrate photoreceptor cells is not widely used in other cell types. In contrast, the retina of higher invertebrates, such as squid, offers an equally accessible transduction system, which uses the widespread second messenger chemistry of an increase in cytosolic calcium caused by the production of inositol-(1,4,5)-trisphosphate ($InsP_3$) by the enzyme phospholipase C, and which may be a model for store-operated calcium influx.

In this article, we highlight some key aspects of invertebrate visual transduction as elucidated from the combination of biochemical techniques applied to cephalopods, genetic techniques applied to flies, and electrophysiology applied to the horseshoe crab. We discuss the importance and applicability of ideas drawn from these model systems to the understanding of some general processes in signal transduction, such as the integration of the cytoskeleton into the signal transduction process and the possible modes of regulation of store-operated calcium influx.

Index Entries: Store-operated calcium influx; phospholipase C; visual transduction; unconventional myosin; G-protein; rhodopsin.

Abbreviations: CaM, calmodulin; CDP, cytidine diphosphate; CDS, CDP-diacylglycerol synthase; DAG, diacylglycerol; DHP, dihydropyridine; *Dtrp, Drosophila* visual mutant conferring "transient receptor potential" phenotype; DTRP, protein encoded by *Dtrp* gene; *Dtrpl, Drosophila* visual mutant encoding TRP-like protein; DTRPL, protein encoded by *Dtrpl* gene; GDP, guanosine diphosphate; G-protein, guanine nucleotide binding protein; GMP, guanosine monophosphate;

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GRK, G-protein-linked receptor kinase; GTP, guanosine triphosphate; *ina*D, *Drosophila* visual mutant confering "inactivation, no after potential" phenotype; INAD, protein encoded by *ina*D gene; $InsP_3$, inositol-(1,4,5)-trisphosphate; $InsP_3R$, inositol-(1,4,5)-trisphosphate receptor; *norpA*, *Drosophila* visual mutant conferring "no receptor potential" phenotype; *ninaC*, *Drosophila* visual mutant conferring "neither inactivation nor activation" phenotype; NINAC, protein produced by *ninaC* gene; PDZ domain, postsynaptic density protein, disk large, zo-1 domain; PH domain, Pleckstrin Homology domain; PIP_2, phosphatidylinositol 4,5-*bis*-phosphate; PKC, protein kinase C; PLC, phosphoinositide-specific phospholipase C; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SML, sucrose monolaurate (dodecyl-D-fructofuranosyl-D-glucopyranoside); *strp*, squid *(Loligo forbesi)* homolog of *Drosophila trp* gene; sTRP, protein encoded by *strp* gene.

Introduction

In almost all cells, there exist signaling pathways linking cell-surface receptors to the release of stored intracellular calcium (Ca^{2+}) into the cytosol *(1)*. The stimulation of isoforms of phospholipase C (PLC), whether by heterotrimeric G-proteins or through tyrosine kinase-linked receptors, causes the release of Ca^{2+} through inositol-(1,4,5)-trisphosphate ($InsP_3$) gated ion channels on specialized areas of the endoplasmic reticulum (ER). The emptying of these internal Ca^{2+} stores leads to the activation of plasma membrane-bound channels, causing a secondary Ca^{2+} influx into the cell known as "capacitative" or "store-operated" calcium entry *(2)*. Store-operated calcium influx appears to be a general phenomenon, and is important in many physiological and pathological processes *(2,3)*. Research into invertebrate vision has so far provided considerable insight into the molecular mechanisms underlying this important biological phenomenon.

In this article, we will briefly summarize the current knowledge of the biochemistry and physiology of the response to light in vertebrate and invertebrate photoreceptor cells. We will then describe in more detail the protein sequencing-based approach we have used to identify, isolate, and characterize the components of visual transduction in squid photoreceptors, and summarize our current ideas on the integration and control of this transduction cascade. Particularly, we will focus on the Ca^{2+} release mechanisms involved in invertebrate phototransduction and on their use as models of store-operated calcium influx phenomena.

Vertebrate Phototransduction

Vertebrate visual transduction has been intensively studied using both electrophysiology and biochemistry. The attention paid to the photoresponse has been in part owing to its accessibility to both these approaches. Because the retina is a neural tissue, with defined and controllable inputs and measurable outputs, it has been described as "an approachable part of the brain" for physiological study *(4)*. The retinae of amphibians have proven especially useful for physiologists owing to the large size of the photoreceptor cells. For the biochemist, the photoreceptor cells (especially of the bovine retina) offer an easily isolated tissue, readily available in large quantities, which is almost entirely given over to the machinery of one signal transduction cascade. As a result, they have provided the raw material for one of the first G-protein-linked signaling pathways to be characterized biochemically *(5)*, and for the first three-dimensional G-protein structures *(6,7)*.

The photoreceptor cells of the vertebrate retina (the rods and cones) are neural cells, made up of two distinct segments. The outer segment is either cylindrical in shape (in rod cells) or conical (in some, but not all cone cells), and contains a stack of flattened membrane disks (in rods) or invaginations of the plasma membrane (in cones) containing the photosen-

sitive receptor protein, rhodopsin, and other proteins of the signal transduction cascade. The inner segment contains the rest of the cell's organelles and is responsible for neurotransmitter release. The two segments are connected by a thin cilium, which is easily broken by shaking a dissected retina in buffer, enabling the convenient isolation of outer segments for biochemical analysis.

The photoreceptor cells respond to light stimulation with a hyperpolarization of the plasma membrane, i.e., the membrane becomes more negative inside *(8)*. This hyperpolarization is brought about by the closure of nonspecific cation channels in the outer segment of the cell. In the dark, these channels allow Na^+ ions to flow into the cell *(9)*, and a steady ionic state is maintained by the action of an inner segment Na^+/K^+ exchanger, which pumps Na^+ ions out of the cell (the so-called dark current) *(10)*. Illumination closes the outer segment channels *(11)*, but does not affect the inner segment pump, so a net Na^+ efflux and increased membrane polarization results.

The closing of the outer segment ion channels is gated by the level of the second messenger compound cGMP *(12)*. In the dark, the intracellular concentration of cGMP is high enough to keep the cation channels open. When stimulated by light, rhodopsin in the disk membrane triggers its associated G-protein, transducin, to adopt an active conformation. As with other heterotrimeric G-proteins, this activation consists of a structural rearrangement of the α-subunit, caused by the replacement of a bound molecule of GDP by one of GTP, and the subsequent separation of the α-subunit from the βγ-subunit *(6,13,14)*. In its GTP-bound state, transducin α activates a cGMP phosphodiesterase (PDE) by binding to and sequestering its inhibitory PDEγ-subunits *(15)*. The activated PDE then hydrolyzes cGMP, and the ensuing drop in intracellular cGMP concentration causes the outer segment ion channels to close.

The second messenger, which regulates the light-activated cation channels, was for a time a matter of dispute, with intracellular Ca^{2+} concentration also being considered as a candidate. However, definitive electrophysiological experiments on patch-clamps of outer segment membrane and on intact outer segments from frog photoreceptors demonstrated the essential role of cGMP in the electrical response of the photoreceptor cells to light *(16,17)*. However, Ca^{2+} ions (which enter the cell via the outer segment cation channels *[18]*) do have an important role in the inactivation and adaptation of the photoresponse, through modulation of PDE *(19)* and channel *(20)* activity, and through the stimulation of guanylyl cyclase, the enzyme that produces cGMP *(21)*.

The biochemistry and physiology of the response of the vertebrate photoreceptor to light is in many ways unusual. The photoreceptor cells are excited in the dark, when they constantly release neurotransmitter (glutamate) into their synapses with other retinal neurons (horizontal and bipolar cells), and suppressed in the light, an unintuitive and initially unexpected finding *(8)*. The stimulation of a PDE by a G-protein also seems to be a relatively rare signal transduction pathway, having been identified only in photoreceptors and in taste transduction *(22)*. Although accessible and well-characterized, the vertebrate visual system does not make a good model for the study of more common G-protein-linked signaling pathways, such as those causing the release of calcium from intracellular stores.

Invertebrate Phototransduction

Invertebrate visual transduction offers a similarly accessible and well-defined signal transduction pathway, which operates by a different mechanism. Most invertebrate photoreceptor cells differ from their vertebrate homologs in that the rhodopsin-containing membranes are organized in an arrangement of microvilli termed the rhabdome or rhabdomere. Microvillar photoreceptor cells depolarize in response to light, i.e., they become less

negative inside, owing to an influx of cations (principally Na^+ and Ca^{2+}) through plasma membrane channels *(23)*. This depolarization causes release of the neurotransmitter histamine *(24)*. Intracellular Ca^{2+} rises rapidly and immediately after illumination *(25)*, and since this initial Ca^{2+} increase is independent of extracellular calcium levels, it is thought to be released from the submicrovillar cisternae (SMC) *(26)*, a compartment of the endoplasmic reticulum that is known to be a Ca^{2+}-sequestering organelle in many microvillar photoreceptor cells *(27)*.

The invertebrate photoresponse has been studied by electrophysiology, mainly on the ventral nerve photoreceptor of the horseshoe crab, *Limulus polyphemus*, and by biochemical analysis of the photoreceptor membranes of cephalopod molluscs, mainly squid (e.g., *Loligo forbesi*) and octopus (e.g., *Paraoctopus defrini*). An additional fruitful avenue of research has been the powerful combination of electrophysiology and genetics in the fruitfly, *Drosophila melanogaster*. A brief summary of the insight provided by each of these areas of investigation is presented below.

Electrophysiology in Limulus

Pressure injection of $InsP_3$ into *Limulus* ventral photoreceptors mimics the Ca^{2+} release caused by light *(28–30)*. However, as was the case for vertebrates, the nature of the intracellular second messenger responsible for plasma membrane channel gating has been the subject of debate, since there is a body of evidence that suggests that cGMP plays a role in the depolarizing photoresponse. Three distinct light-induced currents have been measured in *Limulus* photoreceptors *(31)*, one of which can be selectively blocked by injections of phosphodiesterase *(32)*. Injection of cGMP into *Limulus* photoreceptors activates a current that resembles that produced by light *(33)*, and excised patches of photoreceptor membrane contain channels that can be opened by cGMP *(34)*. Elevation of cGMP levels in *Limulus* photoreceptors after illumination has been reported *(33)*, but these observations have not been reproduced in other studies *(35)*.

It has been reported that the inhibition of inositol phosphate metabolism *(32)* or buffering of intracellular Ca^{2+} *(36)* blocks another of the three light-induced currents in *Limulus* photoreceptors, which suggests that $InsP_3$ and cGMP work via independent, parallel pathways. However, other reports have argued that the *Limulus* photoreceptor light response can be totally blocked by the injection of sufficient Ca^{2+} buffer *(37)* or by Ca^{2+}/calmodulin-binding peptides *(38)*, suggesting that cGMP may function as part of a single, sequential pathway that critically depends on $InsP_3$-mediated Ca^{2+} release.

Although the *Limulus* photoreceptor is the classical preparation for electrophysiological study of invertebrate vision, owing its large cell size and ease of manipulation, it is a far less differentiated cell than most photoreceptors *(39)* and the complexity of its response to light may not be directly mirrored in other invertebrate systems.

Genetics and Electrophysiology in Drosophila

Genetic evidence in flies supports the idea that $InsP_3$-mediated Ca^{2+} release is essential for phototransduction in microvillar photoreceptors. The phenotype of the *Drosophila norpA* visual mutant provides strong evidence that $InsP_3$ is an essential second messenger, since the *norpA* gene encodes a β-subtype phospholipase C (PLCβ). Mutations in this gene render flies completely blind *(40)*, and photoreceptors from *norpA* mutant flies lack the light-induced inward Ca^{2+} current seen in normal photoreceptors *(41)*. There is further supportive evidence for the essential involvement of an inositol phosphate signaling pathway in insect vision: Mutations in the gene encoding an eye-specific isozyme of the enzyme CDP-diacylglycerol synthase (CDS), which is crucial for the replenishment of PIP_2, result in the depletion of the light-induced current, indicating that the amount of PIP_2 present in the eye is

limiting to the visual response *(42)*. Consistent with these genetic findings is the observation that $InsP_3$ increases in *Drosophila* photoreceptors after illumination *(43)* and the cloning of an eye-specific α_q-type G-protein *(44)*.

Two candidates for the ion channel responsible for *Drosophila* photoreceptor depolarization have been cloned: the TRP-type of cation channel (at least two isoforms, discussed in more detail below) and a cGMP-gated channel, which is expressed in eyes and antennae and shows similar properties to the light-induced current, especially in terms of Ca^{2+} permeability *(45)*. The photoreceptors from mutant flies that have no functioning TRP-type channels lack a light-induced current, which shows that the TRP channels are essential for phototransduction *(46)*. TRP-type channels may be directly activated by Ca^{2+} store depletion *(47,48)*. In the absence of defined mutants, the role of the cGMP-gated channel in *Drosophila* phototransduction remains uncertain.

Biochemistry in Cephalopods

Biochemical studies in cephalopods broadly support the idea that invertebrate vision is critically dependent on PLCβ activation. Several different G-protein types have been identified by toxin labeling in cephalopod retinae *(49–51)*, but cloning and protein chemistry experiments have shown that α_q-type G-protein subunits are the major G-proteins found in the retinae of squid *(52)* and octopus *(53)*. This vast predominance of α_q-type G-proteins over other G-protein subtypes in cephalopod photoreceptor microvilli has been confirmed by immunoelectron microscopic observations *(54)*. Stoichiometric considerations would therefore predict that $InsP_3$ production is the major response to light in the cephalopod retina. The light-induced production of cGMP in squid retinae has been reported *(33)* and refuted *(35)*, but a number of groups *(55–58)* have shown that the concentration of $InsP_3$ increases rapidly in squid retina following illumination. A TRP homolog has been isolated in squid *(59)*, but no cGMP-gated channels have so far been identified in cephalopod retinae. The components of the visual transduction cascade in squid are described in greater detail below.

Other Invertebrate Photosystems

In addition to depolarizing microvillar photoreceptors, some invertebrate retinae contain ciliary photoreceptor cells, which hyperpolarize in response to light. The retina of the scallop eye contains two layers of photoreceptor cells: One layer hyperpolarizes in response to light, and the other depolarizes *(60)*. The depolarizing layer contains rhodopsin coupled to an α_q-type G-protein, and presumably has a signal transduction pathway similar to that of cephalopod molluscs. The hyperpolarizing layer contains a different rhodopsin, which is coupled to an α_o-type G-protein, and which appears to stimulate a cGMP-gated K^+ channel via a guanylyl cyclase *(61)*. This is a distinct pathway to that elucidated in vertebrate retinae, though it appears to have parallels in the photoreceptors of the parietal eye of some lizards *(62)*. Phylogenetic analysis of the scallop rhodopsins indicates that both these pathways predate the divergence of vertebrates and invertebrates *(61)*. This idea is supported by the cloning of melanopsin, a rhodopsin-like protein from the photosensitive pigment cells in frog *(Xenopus laevis)* skin, which shares several features with invertebrate rhodopsins, particularly the likely coupling to α_q-type G-proteins, the use of a tyrosine residue as the Schiff base counterion, and a long cytoplasmic tail *(63)*. The tail has no similarity in amino acid sequence to that found in cephalopod rhodopsins *(64)*, but instead contains multiple potential phosphorylation sites *(63)*. However, the presence of "invertebrate"-type rhodopsins in nonvisual photoreception in vertebrates is supportive of the notion that the "invertebrate"- and "vertebrate"-type rhodopsins (and their respective intracellular signaling pathways) arose from gene duplication events that occurred prior to the divergence of vertebrate and invertebrate animals *(65)*.

Superstructure of the Squid Retina

Unlike most invertebrates, cephalopods have large, simple camera eyes, which are morphologically similar to mammalian eyes. The retina of the squid eye contains a single type of photoreceptor cell, which can be divided into inner and outer segments, functionally analogous to the equivalent parts of vertebrate rods. Electron microscopy of squid retina preparations has revealed that the photoreceptor cell outer segment membrane is invaginated to form a highly ordered structure consisting of close-packed microvilli *(66)*. Actin and myosin have been identified in this compartment by immunochemistry, and it has been proposed that each microvillus contains a central F-actin filament from which radiate myosin-like molecules that extend to the microvillar membrane *(66)*. Disruption of the ordered microvillar structure is reported to occur during the photocycle *(67)*. Additional protein components are apparent from electron microscopy that may be involved with holding the microvilli in an ordered structure *(66)*. A diagram of the superstructure of the squid photoreceptor derived from electron microscopy studies is shown in Fig. 1.

Squid (*Loglio forbesi* or *Nototodarus sloanii*) photoreceptor microvillar membranes can be easily isolated by homogenization of the outer segments in a calcium-free buffer using a close-fitting Potter homogenizer, followed by centrifugation on a sucrose cushion. Nonmembrane-associated components can be extracted from the outer segments by a salt wash of the ruptured photoreceptor cells. The microvillar membranes can be further fractionated by solubilization with a mild nonionic detergent, such as octyl glucoside or sucrose monolaurate *(66)*.

The detergent-soluble fraction of the membrane contains rhodopsin and the subunits of the G protein *(68)*. The detergent-insoluble fraction contains actin, and hence is considered to represent the cytoskeleton and proteins intimately associated with the cytoskeleton. This fraction contains four major proteins that have been identified by protein sequencing as actin, a homolog of the *Drosophila* TRP ion channel, and two proteins (140 and 150 kDa) that have been identified by partial protein and cDNA sequencing to be unconventional myosins (A. C., unpublished observations).

The proteins present in the detergent-soluble and detergent-insoluble fractions of the photoreceptor cells visualized by SDS-PAGE are shown in Fig. 2A. Figure 2B shows recombinant G-protein βγ-subunit on Coomassie-stained SDS-PAGE, and is included to emphasize the γ-subunit, which is difficult to visualize in nonoverloaded gels of photoreceptor preparations.

Cytoskeletal Components of the Microvillus

Unconventional myosins have been identified in a wide range of tissues *(69,70)*. The molecules typically comprise an N-terminal S1 myosin head domain, a central calmodulin binding region, and a variable C-terminal domain, for which the structure and function are either poorly understood or unknown. Preliminary protein and DNA sequence information indicates that the squid retinal 140-kDa protein may be homologous to the two gene products associated with the *Drosophila* photoreceptor *ninaC* mutant *(71)*. The *ninaC* gene products arise from splice variation, have different localization in the photoreceptor cell, and are distinct from other unconventional myosins in containing an additional putative N-terminal kinase domain. Phosphorylation of the 140-kDa squid protein can be achieved by incubation with γ-[^{32}P]ATP, indicating intrinsic kinase activity in squid retinal outer segment preparations (A. C., unpublished observations). Mutational analyses of the 174-kDa gene product of *ninaC* have indicated that its kinase domain is required for normal electrophysiology. However, the critical targets of the kinase are unknown. The myosin head domain and the C-terminal region both appear to be important for subcellular localization of the protein, and it has been suggested that the

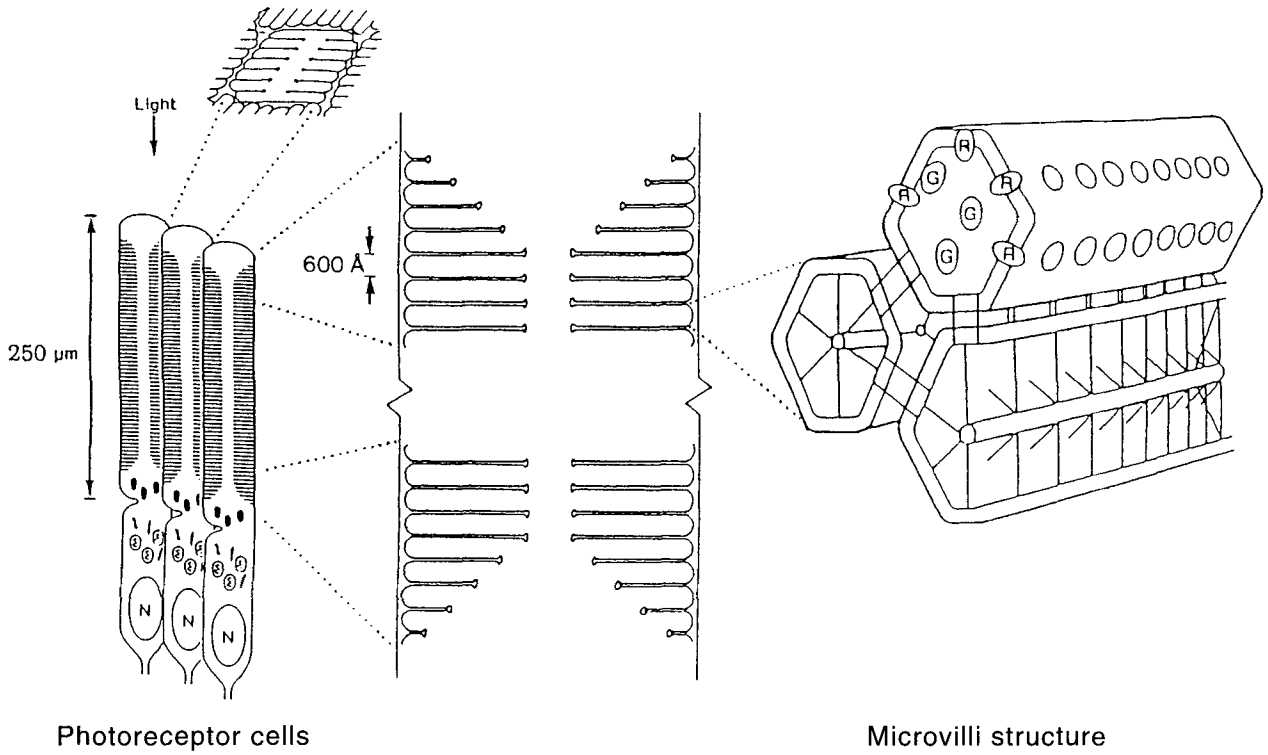

Fig. 1. The superstructure of the squid microvillar photoreceptor. Schematic diagram of the organization and structure of the squid microvillar photoreceptor cells, as deduced from electron microscopy. The central actin filament present in each microvillus and the radial elements, which are presumably made up of a combination of unconventional myosins, are illustrated in the right-hand panel. G = G-protein, R = rhodopsin. Modified from diagrams in refs *(66)* and *(126)*. Reproduced with permission.

function of NINAC is to sequester calmodulin, which may influence Ca^{2+} channel function *(see below)*, although the details of how NINAC functions at the molecular level are not fully understood *(72)*.

The squid retinal 150-kDa protein appears to lack a kinase domain and has a large C-terminal domain of unknown function. DNA and protein sequence data indicate the 150-kDa protein exhibits most similarity in the S1 myosin head region to class V unconventional myosins (A. C., unpublished observation). Class V myosins contain a twin myosin head domain structure, have been associated with cell growth and differentiation, and appear to be involved in the directed transport of molecules to particular cellular locations *(73)*.

200-kDa Protein

Apart from rhodopsin and the TRP-like calcium channel, which are described below, a third major intrinsic membrane protein has been identified within the photoreceptor membrane. This protein is approx 200 kDa in size and is found in the detergent-soluble fraction of the membrane (Fig. 2). Lectin overlay studies indicate that it is glycosylated (N. E., unpublished observations).

Purification and subsequent peptide and DNA sequence analyses have shown that this protein shares regions of sequence identity with the α_2-macroglobulin family of proteins and also revealed the presence of an amino acid sequence motif known as the thioester

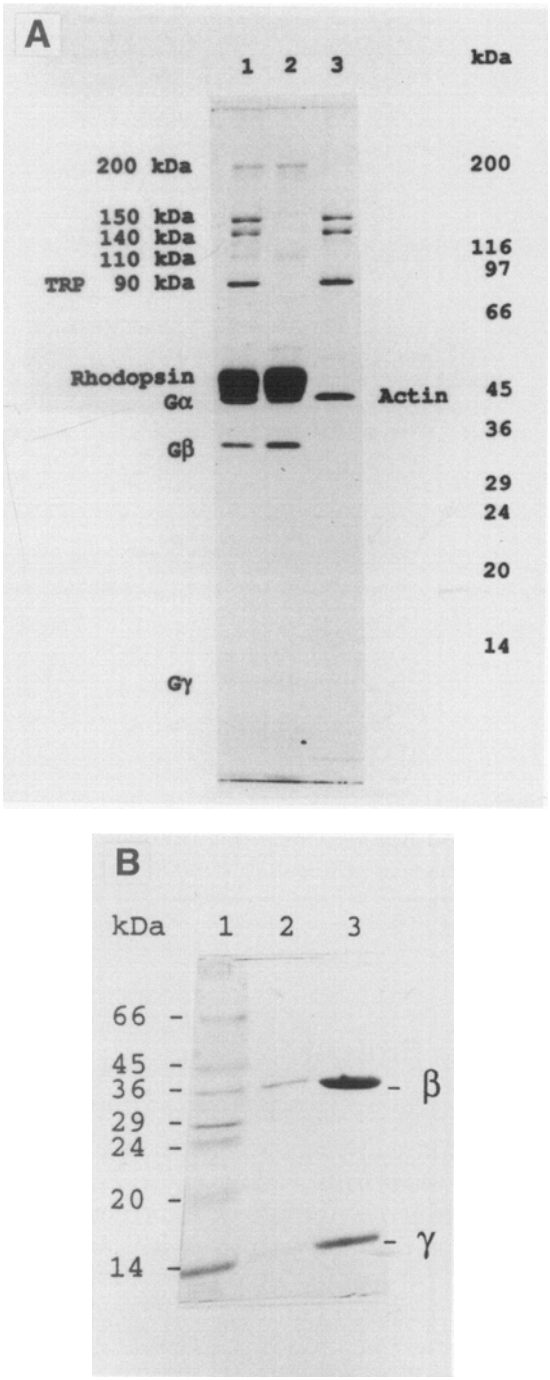

Fig. 2. The major proteins of the squid photoreceptor membrane. **A.** The major proteins in the squid photoreceptor membrane, separated and visualized on a Coomassie-stained 8–18% SDS-polyacrylamide gradient gel. Lane 1: washed microvillar membranes. Lane 2: Proteins solubilized by treatment of membranes with 2% (w/v) sucrose monolaurate (SML). Lane 3: Proteins not solubilized by treatment with 2% SML. **B.** Recombinant squid photoreceptor G-protein βγ-subunit, visualized on a Coomassie-stained 15% SDS-polyacrylamide gel. Lane 1: mol-wt markers. Lanes 2 and 3: Purified recombinant βγ-subunit, expressed in Sf9 cells.

binding region (N. E., unpublished observations). This sequence is highly conserved throughout all known examples of α_2-macroglobulin and complement C3 and C4 molecules from many species. Despite these conserved regions, neither anti-α_2-macroglobulin nor anticomplement C3 antibodies recognized the 200-kDa protein, suggesting that either this protein is a very distant relative of α_2-macroglobulin and complement or that they merely share small regions of sequence similarity, such as the thioester binding region.

However, the presence of this thioester binding region suggests a possible function for this protein. The role of thioester binding regions in other systems is to interact with neighboring hydroxyl or amino groups, forming a covalent linkage with nearby proteins. It is likely that this region has the same function in the squid 200-kDa protein, where it may be involved in crosslinking the microvilli into the observed highly ordered structure *(66)*. Further protein and cDNA sequencing, topology studies, and immunohistochemical localizations are needed to test this hypothesis.

Receptor

Squid rhodopsin shows only a low level of conservation at the amino acid sequence level with respect to vertebrate homologs *(64)*. However, modeling studies *(74)* and more recently electron diffraction experiments *(75)* have shown that the heptahelical structure of these receptors is well conserved between species. In common with rhodopsins from other invertebrate species, squid rhodopsin uses a tyrosine residue rather than a glutamate residue as the Schiff base counterion in binding the 11-*cis*-

retinal chromophore. Both squid and octopus rhodopsins have one striking and distinctive feature compared to all other heptahelical receptors: a long C-terminal cytosolic tail, composed of a repeated proline-rich motif (QGYPP).

The function of the proline-rich tail of cephalopod rhodopsins is not known, though an obvious speculation is that it may be involved in interactions with the cytoskeleton, accounting for the observed low rotational mobility of squid rhodopsin in the membrane compared to vertebrate rhodopsin *(76)*. Two observations indicate that this is probably not the case. First, rhodopsin does not associate with the cytoskeletal fraction after mild detergent solubilization of the photoreceptor membrane (Fig. 2A). Second, removal of the C-terminal tail by specific proteolysis does not increase the rotational mobility of rhodopsin in the membrane as measured by saturation-transfer electron-spin resonance spectroscopy *(76)*. However, the presence of similar proline-rich cytoplasmic tails on a number of complex-forming membrane proteins from a variety of sources *(77)* implies a role for such structures in protein–protein interactions.

Vertebrate and insect rhodopsins are phosphorylated by rhodopsin kinase at serine and threonine residues near the C-terminus of the protein, and an equivalent regulatory phosphorylation is mediated in other heptahelical receptor systems by members of the G-protein-linked receptor kinase (GRK) family *(78)*. Squid rhodopsin contains few obvious phosphorylation sites, and although phosphorylation of squid rhodopsin has been reported, it has proven difficult to reproduce *(49,64)*. However, light- and GTP-dependent phosphorylation of octopus rhodopsin has been observed *(79)* and a specific rhodopsin kinase has recently been purified and cloned from octopus photoreceptors *(80)*. This kinase is interesting, since it is homologous in both sequence and mechanism of action not to vertebrate rhodopsin kinase, but to GRKs of the β-adrenergic receptor kinase type (GRK2/3), i.e., it is translocated to the photoreceptor membrane by the interaction of its Pleckstrin Homology (PH) domain region with the G-protein βγ-subunit.

G-Protein

A number of different studies using toxin-labeling and immunological methods identified a variety of possible G-protein types as the G protein responsible for the transduction of the signal downstream of squid rhodopsin *(49–51)*. However, a combination of protein sequencing and molecular cloning techniques demonstrated that by far the major, if not the only, G-protein α-subunit in the photoreceptor membranes of squid belongs to the toxin-insensitive α_q subclass *(52,68)*, consistent with the idea that the visual transduction process in invertebrates is critically dependent on the stimulation of a PLC. An α_q-type G protein has also been purified from octopus retina and has been shown to be able to stimulate bovine PLC-β *(53)*.

The β- and γ-subunits of the squid G-protein heterotrimer have also been identified and characterized by protein sequencing and molecular cloning *(68,81,82)*. The protein sequence of the β-subunit is very highly conserved in comparison with vertebrate homologs, in contrast to the γ-subunit, which is poorly conserved. The γ-subunit contains an unusual, highly charged N-terminal sequence of five lysines followed by seven glutamates, the function of which is unknown, although lysine-rich sequences have been suggested to form flexible binding sites for inositol polyphosphates *(83)*. A G-protein γ-subunit cloned from *Drosophila* heads has a protein sequence much more similar to mammalian homologs *(84)*. The squid γ-subunit shows a lack of conservation with respect to a number of residues, which seem vital to the interaction between β- and γ-subunits in vertebrates *(7,82)*, which means that the structure of the squid βγ-subunit would provide insight into the recognition processes generally important for the assembly of G-protein βγ dimers.

Phospholipase C (PLC)

Mammalian PLCβs are predominantly membrane-associated proteins, stimulated by G-protein α_q-subunits via a C-terminal region *(85,86)*. They are also stimulated by G-protein βγ-subunits *(87,88)*. It has been suggested that this stimulatory activity may be mediated via the interaction of βγ-subunits with the N-terminal PH domain of the PLC *(89)*, but recently, this activity has been shown to be mediated by another region of the PLC protein, close to the active site *(90,91)*. Despite its central importance for the visual transduction process, the *Drosophila* visual PLCβ, the product of the *norpA* gene, has not been characterized thoroughly with respect to its activation mechanism.

An unusual PLCβ has been cloned from squid retina, which appears to be the only PLC present in this tissue *(92)*. The squid PLCβ is C-terminally truncated and lacks the regions associated with α_q stimulation and membrane association in mammalian homologs, the "G" and "P" boxes, respectively. From its sequence, it would be predicted that the squid PLC would therefore be cytosolic and not stimulated by G-protein α_q-subunits, but rather by G-protein βγ-subunits. The PLC protein has been purified and is indeed not found to be associated with the microvillar membrane, but is found as an ~120-kDa species in the soluble extract of the photoreceptor cells *(92–95)*. However, the soluble PLCβ is sensitive to α_q stimulation *(93–95)*, implying that this enzyme is not activated by α_q-subunits by the same mechanism as mammalian PLCβs. The PH domain of the squid PLC interacts with the squid βγ dimer *(96)*, although this interaction does not appear to activate the enzyme *(94–95)*. The squid PLC also interacts with the C-terminal domain of the sTRP cation channel *(96)*. Both these interactions may serve to localize the PLC to the membrane where it is able to hydrolyze its substrate. The possible synergistic stimulation of the enzyme by both G-protein α- and βγ-subunits, or the regulatory effects of its interaction with the TRP-type ion channel have not been investigated.

Cation Influx Channel

One of the four major proteins found in the detergent-insoluble fraction of the squid photoreceptor membrane is a 92-kDa protein, which was shown by amino acid sequencing to be homologous to the product of the *Drosophila trp (Dtrp)* gene. These protein sequence data were used to clone the squid *trp* cDNA *(strp)* from a squid retina library *(59)*. Analysis of the *Drosophila trp* phenotype gives some indication to the possible role of the sTRP protein in the squid visual signaling pathway.

The *Dtrp* phenotype was first identified on the basis of the observation that certain *Drosophila* were blinded by bright light *(97)*. Studies on *Dtrp* and wild-type flies indicated that the *Dtrp* flies have a much reduced ability to transport Ca^{2+} into the photoreceptor cell *(98,99)*. The cloning and analysis of the *Dtrp* gene *(100,101)* and the related *Dtrpl* (*trp*-like) gene *(102)*, indicate that the *D*TRP and *D*TRPL proteins are integral to the photoreceptor microvillar membrane and are responsible for Ca^{2+} influx. The latter gene, *Dtrpl*, was cloned independently from the *Drosophila* eye on the basis of its ability to bind calmodulin.

*D*TRP has been shown to be a Ca^{2+}-specific channel and is activated by the depletion of calcium stores when expressed in insect cells *(47)* and *Xenopus* oocytes *(48)*. As such, it represents the first store-operated Ca^{2+} channel to be characterized on a molecular level. In contrast, *D*TRPL is constitutively active and shows little cation selectivity when expressed in the same systems *(103,104)*. *D*TRPL is not further activated by store depletion, though further receptor activation increases cation influx. Recent reports indicate that both *D*TRP and *D*TRPL together are responsible for light-induced cation influx. *Drosophila* double *Dtrp/Dtrpl* mutants show minimal cation influx, less than that seen in the *Dtrp* mutant alone, indicating that *D*TRPL contributes to

channel activity. However, *Dtrpl* mutant flies were virtually indistinguishable from wild-type, indicating that the *D*TRP protein is the main influx channel *(46)*. Coexpression of *D*TRP and *D*TRPL in *Xenopus* oocytes gave a novel store-operated current that was not seen if *D*TRP or *D*TRPL was expressed singularly *(105,106)*. Taken together, these studies indicate that both *D*TRP and *D*TRPL are capable of forming channels independently, but in wild-type flies, they may come together to form a multimeric channel. There is also evidence for a third TRP-type protein in *Drosophila (46)*.

The situation in squid appears to be different, since only one TRP-type channel has been identified despite attempts to locate additional TRP-like proteins (J. W. and P. Monk, unpublished observations). Functionally, the sTRP channel may be more similar to *D*TRPL than to *D*TRP. In preliminary electrophysiological experiments, the injection of large amounts (50 ng) of *strp* cRNA into *Xenopus* oocytes results in the quick death of the oocyte, a phenomenon that had previously also been observed for *Dtrpl* cRNA (J. W. and G. Bentley, unpublished observation; *105*). This effect was not seen on injection of the same amount of *Dtrp* cRNA *(105)*, and may therefore be owing to the expression of constitutively active *D*TRPL and sTRP. However, confirmation of these preliminary observations awaits thorough electrophysiological characterization of the sTRP channels, and it remains to be seen what signaling differences exist between *Drosophila* and squid, since there appears to be only one TRP-type protein in squid.

Hydropathy analysis of the sTRP sequence indicates between six and eight putative transmembrane regions *(59)*. The sTRP and *D*TRP channels have limited sequence identity with the transmembrane regions of the dihydropyridine (DHP)-sensitive voltage-gated Ca^{2+} channels *(102)*. The greatest region of identity incorporates the putative pore region. There is also a region of lesser identity between the TRP channels and the proposed voltage sensor region of the DHP-sensitive Ca^{2+} channels. Significantly, most of the series of positively charged residues that make up the voltage sensor are not conserved in the TRP channels, indicating that they are more likely to be ligand- rather than voltage-gated. As with K^+ channels *(107)*, it is thought that four individual sTRP subunits come together to form the functional channel.

On the basis of these data, the topology model shown in Fig. 3 was constructed for sTRP. There are six putative transmembrane regions and a putative pore region. There is a hydrophobic region between transmembrane helices 2 and 3, which has been suggested to form the hydrophobic core of a soluble domain *(108)*. It is not thought that this region forms a seventh transmembrane region, since a number of observations indicate that both the N- and C-termini are located intracellularly. Analysis of the amino acid sequence of sTRP reveals potential glycosylation sites in both the N- and C-terminal domains. However, expression of the channel by in vitro translation in the presence and absence of microsomal membranes indicated that no glycosylation occurred *(59)*. Additionally, the native sTRP protein was not labeled by a panel of lectins (H. Saibil and K. Langmark, personal communication), indicating that it is not glycosylated.

The N-terminal regions of the TRP-type proteins are highly conserved (48% identity between sTRP and *D*TRP), and all contain an ankyrin-like repeat structure. These ankyrin-like regions are also conserved in the more distantly related members of the expanding TRP family of ion channels, such as the recently cloned vanilloid receptor VR1, which functions as a thermal receptor in peripheral nociceptors *(109)*. In red blood cells, ankyrin is known to bind to spectrin, which in turn binds to the actin cytoskeleton *(110)*. This region may therefore play a role in linking sTRP to the cytoskeleton and could explain the fractionation of sTRP with the cytoskeletal proteins. It is also possible that there may be an interaction with the ankyrin binding site of the $InsP_3R$ *(111,112)*. As yet, no regulatory role has been

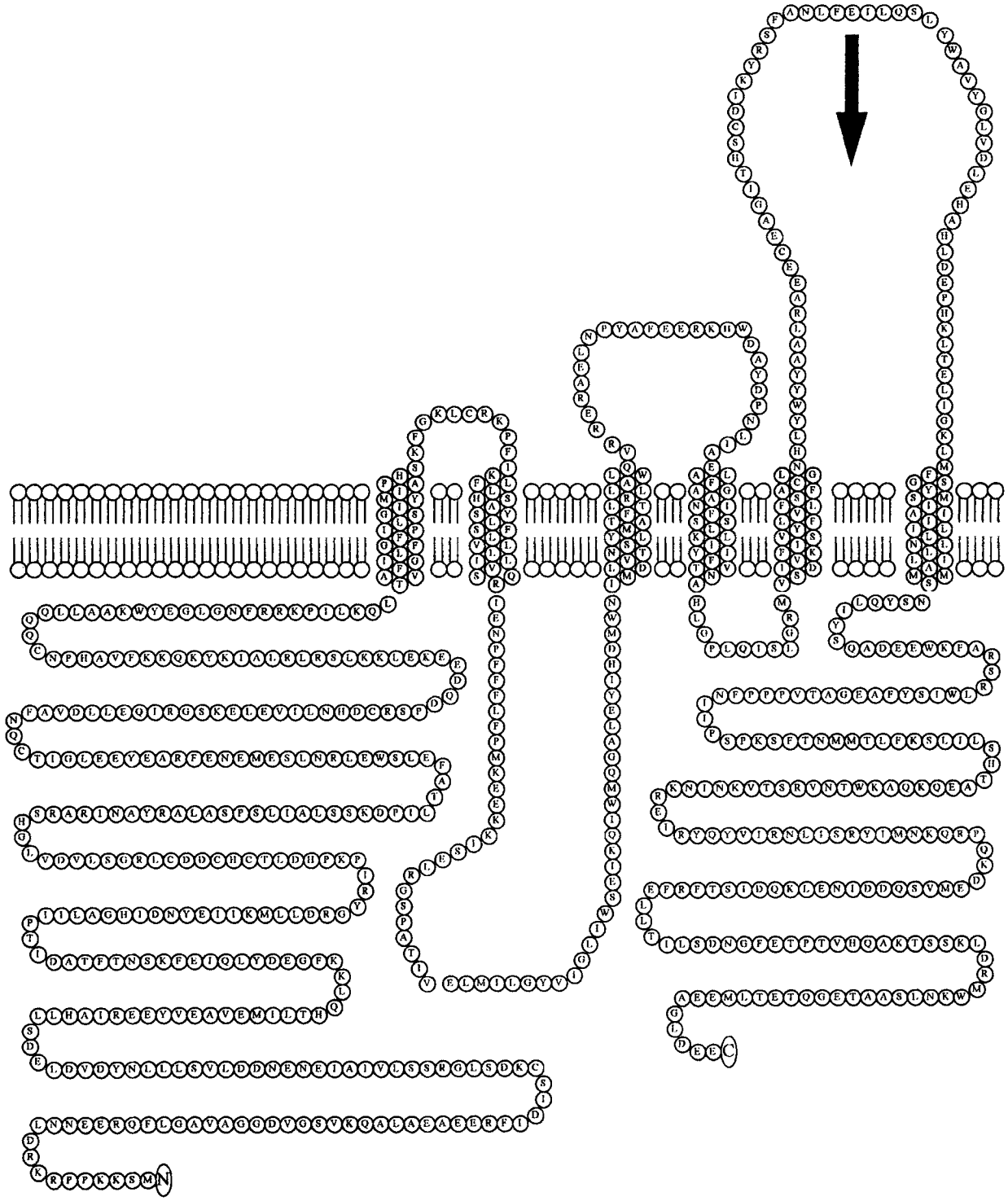

Fig. 3. Topological model of sTRP. A model of the membrane topology of the sTRP channel *(59)*, based on hydropathy analysis, homology to K^+ channels and in vitro glycosylation data. The large extracellular loop marked with an arrow probably constitutes the pore-forming region, as seen in K^+ channels. Reproduced with permission from ref. *59*.

shown for the N-terminus; rather, it appears the C-terminus is crucial for the regulation of the channel.

The C-terminal domain of all TRP proteins contains a number of regions that may contribute both structurally and functionally to the channel. *D*TRP, *D*TRPL, and sTRP have all been shown to bind calmodulin *(59,102,113)* via the C-terminal domain. Apart from this site, there is very little domain conservation between the various C-terminal domains, and they vary greatly in length. It therefore seems probable that differences in regulation between the TRP proteins are owing to the differences between the C-terminal regions. This has been shown to be the case for the *Drosophila* TRP-type proteins through a series of domain swap experiments *(114)*, which show that the elongated C-terminus of *D*TRP could confer sensitivity to store emptying onto *D*TRPL, without affecting channel selectivity.

Possible Regulatory Mechanisms of TRP Channels

The mechanism by which store emptying leads to calcium influx is unknown, though two main hypotheses have been put forward *(3)*: (1) the stores are physically linked to the influx channel allowing direct control of ion influx, or (2) a diffusible messenger is released from the stores, triggering the plasma membrane channel directly or indirectly by activating a further signaling pathway.

The direct link hypothesis proposes that the $InsP_3R$ physically triggers an influx of calcium via *D*TRP *(3,115)*. This hypothesis requires *D*TRP to be situated at the base of the microvillar membrane close to the intracellular calcium stores in the submicrovillar tubule. Initial reports *(116)*, carried out on *Drosophila* pupae, showed that this is indeed the case; however, two recent studies *(46,113)* have demonstrated that in adult flies, *D*TRP is distributed throughout the microvillar membrane. This does not preclude an interaction between *D*TRP and the $InsP_3R$ at the base of the microvillar membranes, but does indicate that this is not the primary regulatory mechanism for the majority of *D*TRP. Initial studies in squid, using a gel overlay assay, have not shown a link between sTRP and a putative squid $InsP_3R$ (J. W., unpublished observation). Further investigation is required to rule out completely any interaction between these two proteins.

The $InsP_3R$ is not the only protein that may be regulating sTRP by a direct protein–protein interaction. The prominent cytoskeletal network of the photoreceptor appears to be linked directly to the sTRP protein, indicating that it may regulate the function of the channel. Preliminary solubilization studies indicate that sTRP may be interacting with the 150-kDa unconventional myosin, which in turn binds to actin (J. B. C. F. laboratory, unpublished observations).

Several diffusible regulators are also candidates for the regulation of TRP-like channels. The invertebrate TRP proteins have all been shown to bind calmodulin (CaM) at their C-termini, which may modulate channel function. However, the binding varies between each of the TRP proteins: *D*TRP has a single, Ca^{2+}-independent CaM binding site *(112)*, whereas *D*TRPL has two, one of which is Ca^{2+}- and kinase-dependent, and the other, which binds CaM only in low-Ca^{2+} concentrations *(117)*. CaM has been shown to bind to both the native sTRP protein and the recombinant C-terminal domain in a Ca^{2+}-dependent fashion. The NINAC proteins of *Drosophila* are known to bind CaM, and it has been proposed that they act to localize the CaM in the rhabdomere *(118)*. The squid 140-kDa unconventional myosin, which also binds CaM, may serve a similar purpose.

*D*TRPL has also been shown in some patch-clamp studies to be directly activated by q-type G-protein α-subunits *(119)*. The possibility remains open then, that TRP-type channels (including sTRP) may be directly regulated by $InsP_3$ *(120)*, $G\alpha_q$ *(119)*, PLC *(96)*, CaM (59,113,117), or a varying combination of these different factors in different cellular locations and circumstances *(108)*. Clearly, much work remains to be done before a comprehensive

picture of the complex regulation of these important channels is achieved.

The Phototransduction Complex

Evidence now exists that important components of the squid visual transduction cascade are associated into a functional complex that is linked to the cytoskeletal network in the photoreceptor cell microvillus. The four principal components of the squid retinal microvillar membrane, which are not solubilized under nondenaturing conditions, are tightly associated with each other (Fig. 1A, lane 3). Both unconventional myosins presumably remain bound to the central actin core of the microvillus, and of the known integral membrane proteins present in the photoreceptor membrane, only the sTRP cation channel partitions with the cytoskeleton. Solubilization experiments appear to indicate that the partitioning of the channel with the cytoskeleton represents a specific protein–protein interaction rather than a nonspecific precipitation event (A. C. and J. B. C. F. laboratory, unpublished observations). This specific partitioning of the cation channel with the cytoskeletal components is of particular interest, since it implies a functional interaction between mechanical (actin-myosin motor) and electrophysiological (cation channel) components of the cell. The observation that the cytoskeleton of the microvillus undergoes rapid change in response to light supports this idea *(67)*, but the precise biological significance of this interaction to the photoresponse is still unclear. One immediate speculation is that this represents a mechanical component of adaptation of the photoresponse, perhaps driven by large calcium influx in strong illumination, but this remains to be tested.

The identification of the unusual squid PLCβ as an interacting partner with both the sTRP channel and with the G-protein βγ-subunit *(96)* also implies that the immediate regulation of the phototransduction cascade may occur via a transduction complex arranged around the cytoskeletally tethered channel. Recent evidence from studies on *Drosophila* and *Calliphora* indicate that many components of the visual transduction cascade in flies are spatially localized into a so-called transducisome by interaction with the PDZ domains of the *inaD* gene product *(113,121–123)*. PDZ domain-containing proteins appear to have a general role in biological systems as organizers of membrane receptors and channels *(124)*, but we have no evidence of a protein in the squid photoreceptor membrane that performs the membrane organizational function of INAD in *Drosophila*. Together, these observations lead us to speculate that although the signaling apparatus in squid may indeed be organized into a discrete membrane-bound complex analogous to that seen in flies, it is assembled via a distinct mechanism. Our current model of the squid transducisome is shown in Fig. 4.

Functional studies on the components of this complex are now required to explore fully the regulatory significance of such a tightly physically integrated calcium influx system. Specifically, it is important to understand the functional consequences of the association of the soluble PLC with the sTRP ion channel and with the G-protein βγ-subunit. A recent paper has demonstrated that the correct localization of PLC to the signaling complex in *Drosophila* visual transduction is important for efficient function *(125)*. Analysis of the functional consequences of the interactions made by the PLC in the squid visual transducisome may reveal new mechanisms for the control of calcium influx systems.

Note Added in Proof

The recent identification of a transducisome in mammalian rod photoreceptors, which is mediated by the interaction of glutamic-acid-rich proteins with cGMP PDE and guanylyl cyclase (*127*), highlights both the importance of macromolecular complex formation in rapidly acting signaling pathways, such as visual transduction, and the variety of ways in which such complexes can be assembled.

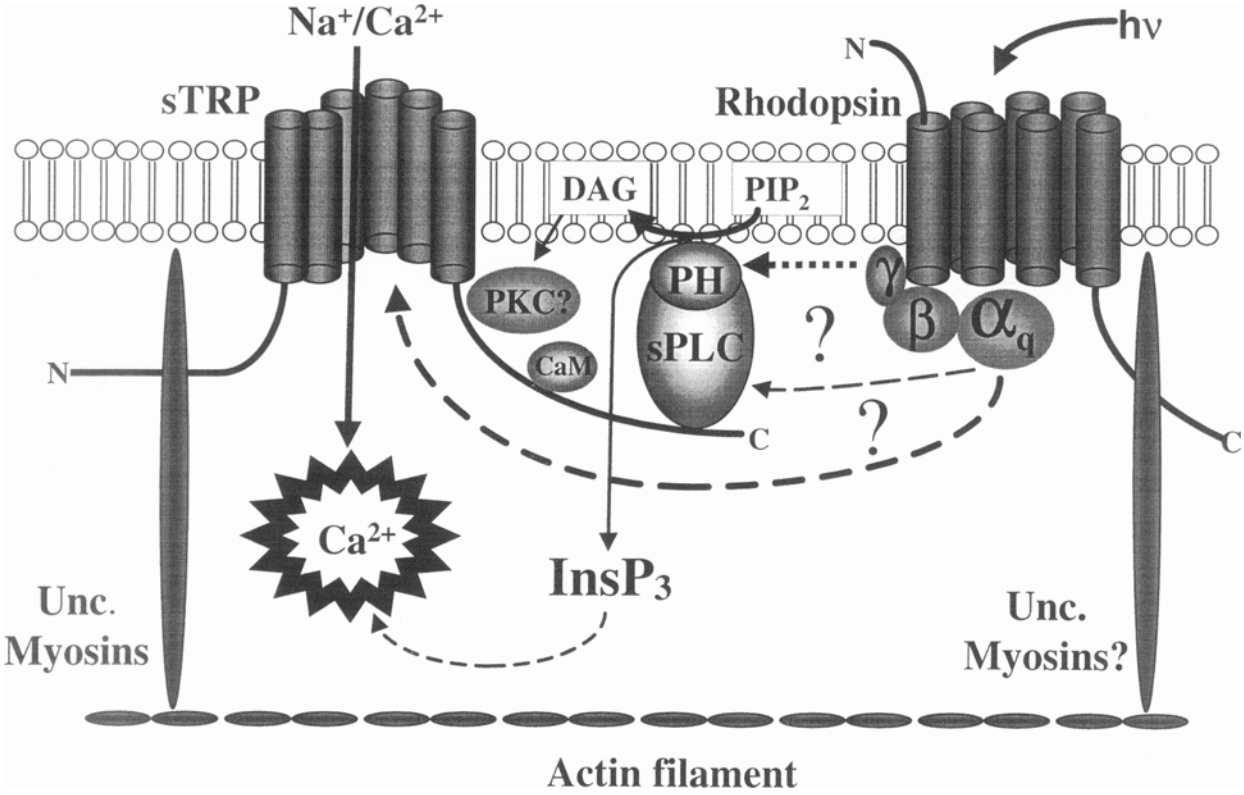

Fig. 4. Model of the membrane organization of the photoreceptor complex. This model illustrates the key role played by the cytoskeleton in organizing the functional components of squid phototransduction, in particular the sTRP channel. The central role of PLC in the signal transduction process, and its physical integration with the sTRP channel are highlighted. The main unanswered questions about the functioning of the complex are labeled with question marks: The mechanism of recruitment of the PLC to the membrane and the details of its functional modulation by G-proteins and other components, and the mechanism of gating and regulating the sTRP channel.

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