REGULATION OF THE TRP CALCIUM CHANNEL BY EYE-PKC IN *DROSOPHILA*

By

Daniela Catalina Popescu

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Approved:	Date:
Bih-Hwa Shieh, Ph.D.	08/03/06
Brian E. Wadzinski, Ph.D.	08/03/06
Louis J. DeFelice, Ph.D.	08/03/06
David L. Hachey, Ph.D.	08/03/06
John H. Exton, M.D., Ph.D.	08/03/06

To my parents, Ioana and Ioan, for their infinite love and support.

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Arr = arrestin

CaMK = Ca^{2+}/ calmodulin-dependent kinase

CID = collision induced dissociation

DAG = diacylglycerol

ERG = electroretinogram

GPCR = G-protein coupled receptor

GST = glutathione S-transferase

G_t = transducin

hINADL = human INAD-like

inaC = inactivation-no-afterpotential C

INAD = inactivation-no-afterpotential D

IP_3 = inositol trisphosphate

LC-MS = liquid chromatography-mass spectrometry

MALDI-TOF = matrix-assisted laser desorption ionization time-of-flight

NHERF = Na^+/ H^+ exchange regulatory factor

NINAA = neither inactivation nor afterpotential A

NINAC = neither inactivation nor afterpotential C

NORPA = no-receptor-potential A

PDK-1 = phosphoinositide-dependent kinase-1

PDZ = Postsynaptic density 95; *Drosophila* Discs large; and Zonula occludens 1

PIP_2 = phophatidylinositol-4, 5-bisphosphate

PKC = protein kinase C

PLC = phospholipase C

pRGCs = photosensitive retinal ganglion cells

PUFAs = polyunsaturated fatty acids

RACK = receptor for activated C-kinase

RICK = receptor for inactivated C-kinase

S.D. = standard deviation

S.E.M. = standard error of the mean

Sf = *Spodoptera frugiperda*

SOC = store-operated channel

TRP = transient receptor potential

TRPL = TRP-like

WB = Western blotting

wt = wild-type

CHAPTER I

INTRODUCTION

Visual transduction

Living on Earth can be a challenge for most creatures, and therefore, organisms endowed by evolution with advanced senses are best able to survive. To survive, an organism needs to accomplish three goals: eat, mate, and protect itself from predators and disease. In a world of light-dark cycles, the sense that provides the highest flux of information to the brain in the most advanced organisms is vision. Light represents energy in the form of electric and magnetic fields, which is converted to impulses in the brain. There are different types of light, which are classified according to wavelength (Figure 1). Humans are able to perceive light in wavelengths ranging from 400 nm (seen as violet) to 700 nm (seen as red). Birds and insects can see ultraviolet (UV) light that ranges from 200 nm to 400 nm, an advantage, since many seeds, fruits, flowers and even bird's plumage contrast with their background better in UV than visible light, improving the chance of finding food and mating. In addition, there are reptiles that can see infrared light, which helps them identify warm-blooded prey at night. The ability to see different types of light relies on the existence of different photoreceptor cells. Photoreceptor cells are highly specialized neurons that absorb a unit of light (a photon) and then, relay this information to other neurons through neurotransmitters, which trigger a change in their membrane potential. The process by which a light stimulus generates an electrical response is called visual transduction or phototransduction. Visual transduction is initiated by activation of light-sensing receptors, which are composed of seven transmembrane proteins called opsins that are coupled to a derivative of vitamin A,

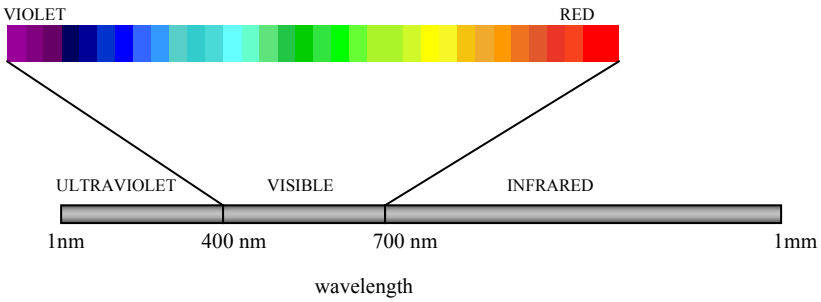


Figure 1. Part of the electromagnetic spectrum. The human eye can perceive light in the visible domain with wavelengths between 400-700 nm, and maximum absorption at 510 nm, a wavelength that corresponds to the color green. Birds and insects can see ultraviolet light (wavelength < 400 nm), whereas reptiles can see infrared light (wavelength > 700 nm).

retinal, which is the light absorbing pigment molecule. Light leads to isomerization of the retinal chromophore, which determines the conversion of the light-sensing receptor from its inactive to its activated form. Activated receptors trigger a cascade of intracellular signaling events, which eventually leads to generation of a nerve impulse that is transmitted to the brain by the optic nerve.

Vertebrate visual signaling

The vertebrate retina contains three types of photoreceptor cells: rods, cones and photosensitive retinal ganglion cells (pRGCs). Rods are responsible for night vision, whereas cones are responsible for daylight vision and color perception. pRGCs represent a small population (less than 3%) of neurons within the RGC layer of the retina that detects the environmental brightness (irradiance) (*1*). Rhodopsin is the light-sensing receptor in rods, photopsins are the light-sensing receptors in cones, and melanopsin (*2*) has been recently shown to be the receptor that senses light in pRGCs (*3*).

In rods and cones, light triggers the activation of rhodopsin or photopsin, from its inactive to its activated form, which in turn activates a heterotrimeric G-protein, transducin (G_t) (Figure 2A). The GTP-bound form of the α subunit of G_t stimulates a cGMP-phosphodiesterase that hydrolyzes cGMP to GMP, which leads to closure of cGMP-gated Na^+ and Ca^{2+} channels in the plasma membrane (*4*). Because these cations can no longer enter, the net result is hyperpolarization of photoreceptors in response to light (Figure 2A).

It has been recently shown that, unlike the visual signaling cascade of rods and cones, pRGCs utilize a pathway that is closely related to the invertebrate visual signaling

cascade. Activation of vertebrate melanopsin by light appears to trigger a *Drosophila*-like signaling cascade, involving Gq, phospholipase C and protein kinase C (*5*) (see below), which leads to depolarization of the photoreceptor cell (*6-8*).

Drosophila visual signaling

Drosophila visual signaling is a G-protein coupled signaling pathway initiated by activated rhodopsin, metarhodopsin, which activates a heterotrimeric Gq protein. Specifically, Gq_{α} stimulates a phospholipase C β (PLCβ) encoded by the fly no-receptor-potential A (*norpA)* locus (*9*) (Figure 2B). NORPA hydrolyzes phophatidylinositol-4, 5-bisphosphate (PIP_2) to generate inositol 1, 4, 5-trisphosphate (IP_3) and 1, 2-diacylglycerol (DAG) leading to opening of the transient receptor potential (TRP) and TRP-like (TRPL) cation channels, which results in depolarization of the photoreceptor cell (*10*).

Why *Drosophila*?

Drosophila melanogaster (Figure 3A) is a small organism that is easily reared in the laboratory, and has a short life cycle of about two weeks. *Drosophila* has proven to be an attractive system for understanding the rules of genetic inheritance as well as the molecular mechanisms underlying various processes, such as circadian rhythm and development. Forward genetic analysis using ethyl methane sulfonate-induced mutations has been used classically for identification of various mutants based on their defective phenotypes (*11*). Furthermore, since the *Drosophila* genome is small (less than a tenth of that of humans and mice), and has been entirely sequenced, mutant genes can now be readily identified. P-element-mediated germline transformation is a tool unique to *Droso-*

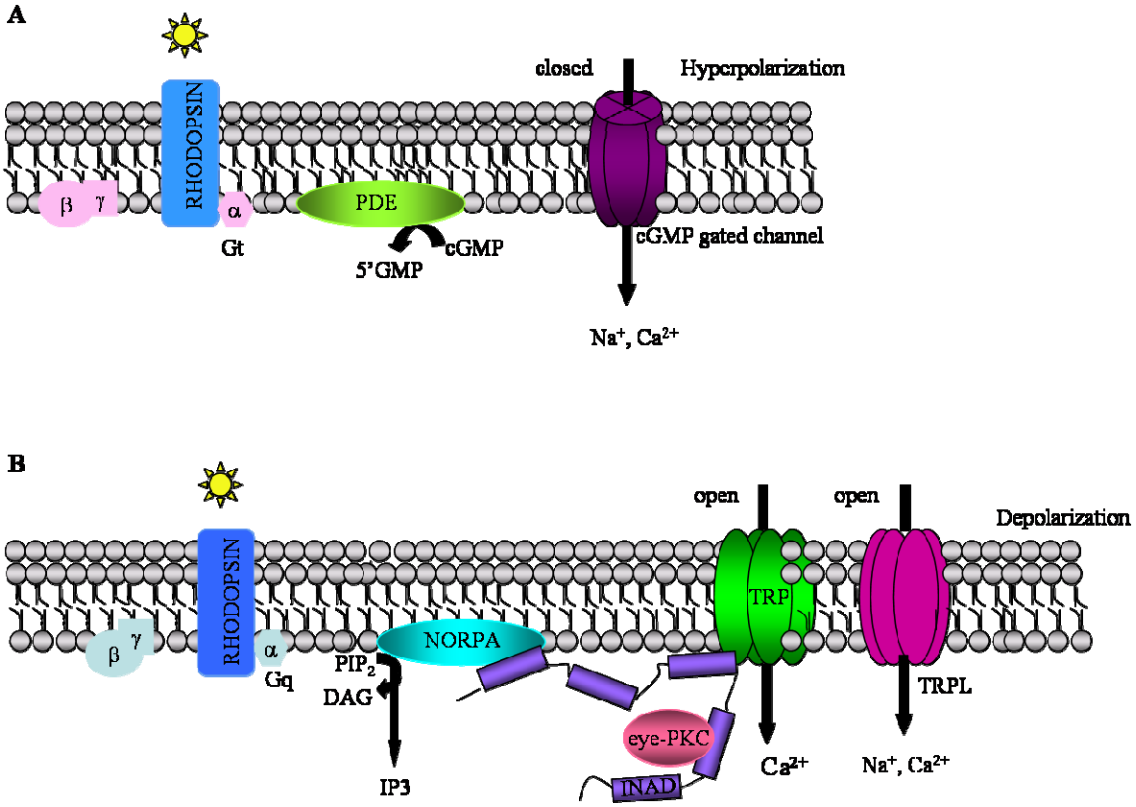


Figure 2. Molecular basis of visual signaling. (**A**) Vertebrate rods and cones hyperpolarize in response to light due to the closure of cGMP-gated cation channels. (**B**) Vertebrate pRGCs utilize a *Drosophila*-like visual signaling pathway. Shown is a cartoon depicting *Drosophila* visual signaling, in which light triggers sequential cellular events that lead to opening of TRP and TRPL cation channels, and subsequently, depolarization of the photoreceptor cell. See text for details.

phila that is used for incorporating genes of interest in the genome to generate transgenic flies (*12*). Transgenic flies can be used to characterize the function of a gene product in its native environment, using cellular, biochemical and electrophysiological analyses. Studies performed in flies have provided useful information for understanding the structure and development of the visual system. Moreover, the pathogenesis of retinitis pigmentosa, a hereditary human disease characterized by degeneration of photoreceptor cells in the retina, has been better understood following studies performed in flies. The first genetic defect associated with human recessive retinitis pigmentosa was discovered to be similar to a mutation in the *Drosophila* rhodopsin gene: this mutation results in premature termination of the opsin protein at the third cytoplasmic loop, and causes recessive retinal degeneration in flies (*12*).

Drosophila was also the first organism in which a TRP channel was discovered. *trp* flies showed behavioral deficits at high levels of ambient light (*13*). Further molecular and electrophysiological analyses revealed that *Drosophila* TRP is a Ca^{2+} channel essential for fly vision. Many vertebrate and invertebrate TRP channels have been identified, and they constitute the TRP channel superfamily. Some vertebrate TRPs mediate responses to nerve growth factor, pheromones, temperature, mechanical stimuli, osmolarity, vasodilators, and metabolic stress (*14*). In addition, pathophysiological conditions appear to involve TRP channels. Among these are hypomagnesemia and hypocalcemia that result from a defective TRPM6 channel, autosomal dominant polycystic kidney disease caused by mutations in TRPP2 or TRPP1, and mucolipidosis caused by mutations in TRPML1 (*15*). All of these TRP proteins have related counterparts in *Drosophila.* Because basic cellular mechanisms and proteins are

conserved across species, understanding the regulation of these TRP channels in *Drosophila* can give us insight into similar regulation mechanisms present in humans.

Recently, another type of photoreceptor cell has been discovered in human (*3*). This class of photoreceptor cell, pRGC, is localized within the RGC layer of the retina, and detects the environmental brightness (irradiance) (*5*). Interestingly, pRGC utilizes a signaling pathway similar to that of *Drosophila* visual signaling, in which light leads to depolarization of the photoreceptor cell, and not to the hyperpolarization seen in vertebrate rods and cones. The signaling pathway in pRGCs includes Gq, PLC and PKC; however, the details concerning phototransduction mechanism in pRGCs remain elusive. Therefore, elucidating *Drosophila* visual signaling can provide a better understanding of visual signaling in pRGCs.

The visual system of *Drosophila*

Drosophila, as with any dipteran insect, has compound eyes, each of which contains approximately 800 unit eyes or ommatidia (Figure 3B) (*10*). Each ommatidium is composed of eight photoreceptor neurons (R1-R8) and 12 accessory cells. Photoreceptor neurons have a particular spatial distribution within each ommatidium, with R1-R6 occupying the peripheral region (outer photoreceptors), and R7-R8 residing in the central region of the ommatidium (central photoreceptors) (Figure 3C). Each photoreceptor cell possesses specialized organelles called rhabdomeres. A rhabdomere contains approximately 60,000 microvilli, which are important for maximizing light absorption (Figure 3D). The rhabdomere of a photoreceptor neuron is important for visual transduction because it contains the proteins involved in visual signaling.

Major components of *Drosophila* visual signaling

Rhodopsin

The sensitivity of the photoreceptor cell to light of a particular wavelength is determined by the type of rhodopsin it contains. Rhodopsin is a G-protein coupled receptor (GPCR) which consists of a seven-transmembrane protein called opsin that is covalently attached at its seventh transmembrane helix to the vitamin A derivative called retinal, or 3-hydroxy-retinal. Light triggers the 11-*cis* to *all-trans* photoisomerization of retinal, resulting in conversion of rhodopsin to its activated form, metarhodopsin. In *Drosophila*, there are six types of rhodopsin with distinctive spectral sensitivities. Rhodopsin 1 is the major rhodopsin in the retina, present in the six outer photoreceptor neurons, R1-R6, which are sensitive to blue light (*16*). Rhodopsin 2 is present in the ocelli, which are visual organs located on the top of the head, important for visual guidance of the fly. Rhodopsin 3 is expressed in R7 and R8, conferring sensitivity to UV-light, whereas rhodopsin 4 is another UV-sensitive rhodopsin present in R7 neurons. Rhodopsins 5 and 6 are present within R8, conferring sensitivity of these neurons to blue or green light, respectively. There are three possible combinations of R7/R8 in the retina that permit different spectral sensitivities (*17*). 70% of total R7/R8 combinations is composed of rhodopsin 4 in R7 / rhodopsin 6 in R8, whereas 30% consists of rhodopsin 3 in R7 / rhodopsin 5 in R8 (*10*). These two combinations allow color discrimination over a broad range of light wavelengths. Another combination (rhodopsin 3 in R7/ rhodopsin 3 in R8), present in a row of ommatidia in the most dorsal part of the eye (the dorsal rim area), detects polarized eye and is thus important for the navigation of the fly (*17*).

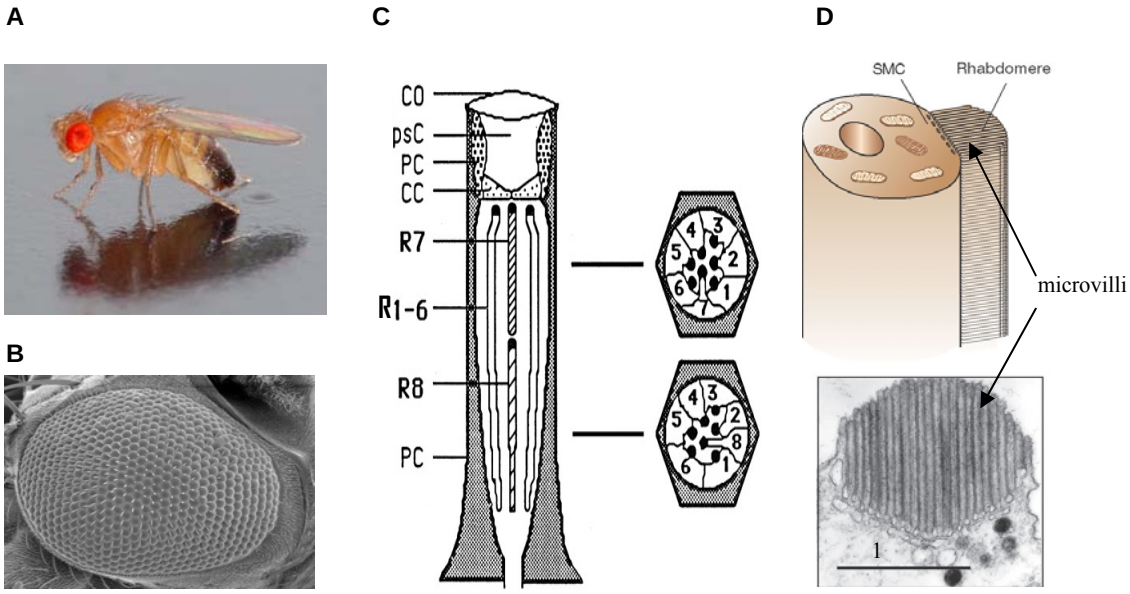


Figure 3. The compound eye of *Drosophila.* (**A**) *Drosophila melanogaster*, lateral view (image from http://en.wikipedia.org/wiki/Drosophila_melanogaster). (**B**) Scanning electron micrograph of the compound eye. (**C**) The adult ommatidia. CO, corneal lens cells; psC, pseudocone; PC, pigment cells; CC, cone cells; R, photoreceptor cell. Transverse section of proximal (*lower*) and distal (*upper*) regions of the ommatidia are shown on the right. Adapted from Montell, 1999. (**D**) The photoreceptive membrane of a photoreceptor cell. Sections through a rhabdomere are shown in both the upper panel and the bottom elecron micrograph. Each rhabdomere contains approximately 60,000 microvilli. SMC, submicrovillar cisternae, are presumed to be smooth endoplasmic reticulum Ca^{2+} stores. Adapted from Hardie and Raghu, 2001.

As with all GPCRs, the opsin of the major rhodopsin (rhodopsin 1) translocates to the rough endoplasmic reticulum (ER), following synthesis on membrane bound ribosomes, where it undergoes proper folding and assembly. Two proteins, NINAA and calnexin, are essential for the posttranslational processing of opsin. NINAA is a cyclophilin homolog (*18*) that is important for the export of opsin from the ER, since mutations in *ninaA* lead to a dramatic retention of opsin in the ER cisternae (*19*). In addition, calnexin is also critical for biosynthesis of opsin, as mutations in calnexin result in defects in opsin maturation, and age-dependent retinal degeneration (*20*).

The newly synthesized opsin incorporates the chromophore retinal (*21*), and the resulted rhodopsin molecules are transported through the secretory pathway to the microvilli of rhabdomeres (Figure 3D), where phototransduction takes place.

Gq

In the fly eye, light triggers the conversion of rhodopsin to metarhodopsin, which in turn, stimulates the heterotrimeric Gq protein. Following GDP-GTP exchange, GTP-bound $G\alpha_q$ is released from $G\beta\gamma$ and transmits the activation signal to PLCβ (NORPA). $G\alpha_q$ acts as a shuttle between metarhodopsin and NORPA that is associated with the INAD signaling complex (*22*). The essential role of $G\alpha_q$ in phototransduction was revealed in flies lacking $G\alpha_q$, which exhibited a severe loss of light responsiveness (*23*). $G\alpha_q$ has been shown to undergo light-dependent translocation from rhabdomeres to the photoreceptor cell body (*24*). This process appears to be an efficient way of controlling the number of $G\alpha_q$ molecules available for signaling, and thus may play a role in light-adaptation (*24*).

$G\alpha_q$ is two-fold less abundant than Gβ in photoreceptor cells, and the excess of Gβ has been shown to be important for preventing spontaneous activity of photoreceptor cells in the dark: mutant flies with lower levels of Gβ displayed a dramatic increase in spontaneous activity of photoreceptor cells in the absence of light (*25*).

NORPA

NORPA is a PLCβ essential for *Drosophila* visual signaling (*9*). At its N-terminus, NORPA contains a membrane/phospholipid-binding pleckstrin homology (PH) domain and an EF-hand calcium-binding domain. These domains are followed by the catalytic domain (X and Y), which is responsible for the PLC activity. At its C-terminus, NORPA has a C2 domain which is involved in Ca^{2+} binding, and a Gq-binding domain important for its interaction with Gq. In addition, NORPA contains a PDZ-interacting domain at its C-terminus, important for its interaction with the scaffolding protein inactivation-no-afterpotential D (INAD).

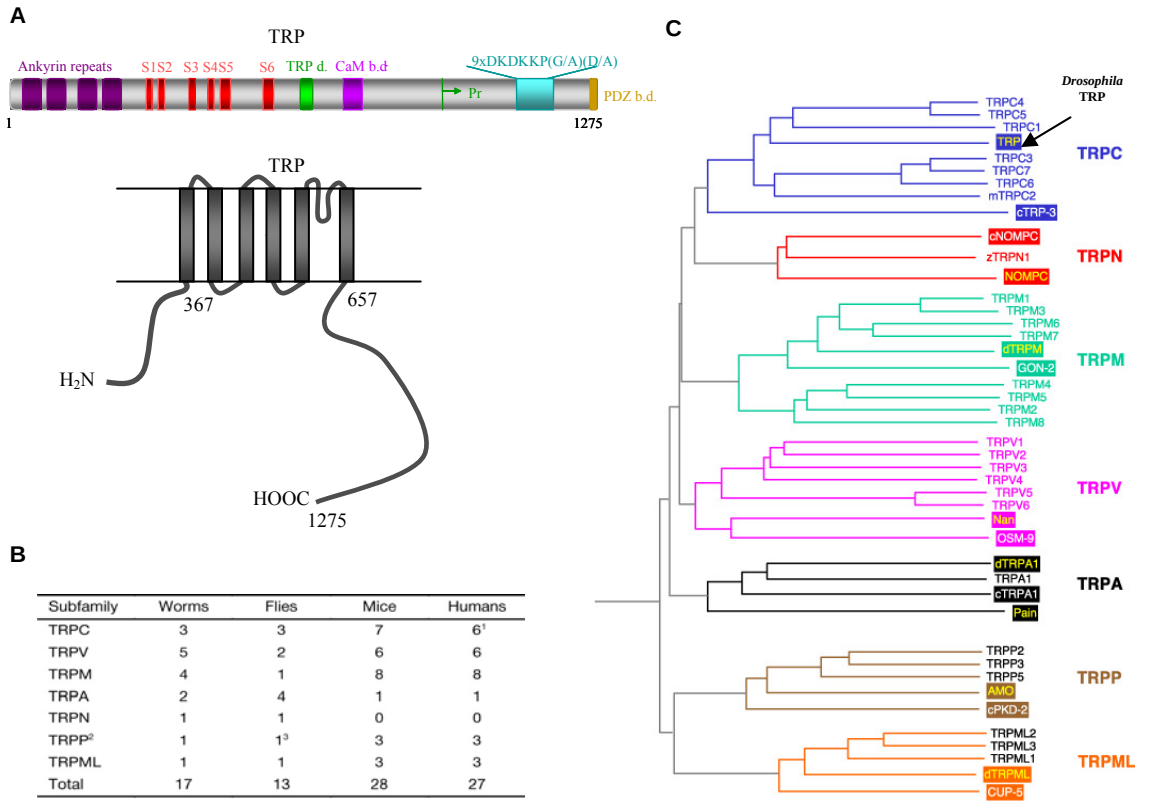
As mentioned above, activated NORPA catalyzes the hydrolysis of PIP_2 to generate IP_3 and DAG, which leads to opening of the TRP and TRPL cation channels, and subsequent depolarization of the photoreceptor cell (*26, 27*). The key second messenger that activates the TRP channel is generated upon NORPA stimulation, and is thought to be either DAG or its lipid metabolites (*28, 29*), whereas IP_3 does not appear to play a role in TRP activation (*10, 30*). DAG may have a dual function, since it also activates the eye-specific conventional PKC (eye-PKC) that is vital for deactivation of the light response (*31, 32*).

NORPA shares greatest sequence identity (49%) with mammalian PLCβ_4. PLCβ_4 is expressed in mammalian retina, cerebellum and brain regions that are involved in the visual pathway, including the lateral geniculate nucleus and superior colliculus (*33*). Mice lacking PLCβ_4 have impaired visual processing abilities, as revealed by behavior tests, and display abnormal electroretinogram recordings. However, isolated rod cell recordings show no apparent defect in rod signaling pathway, suggesting that PLCβ4 is important for visual signaling processing, and not for the initial step of phototransduction (*33*). Whether PLCβ4 is expressed in pRGCs of retina for detecting the environmental brightness remains to be determined by future studies.

The TRP Ca^{2+} channel

The *Drosophila* TRP Ca^{2+} channel plays a crucial role in the visual signaling pathway. Originally, the name transient receptor potential (*trp*) described a mutant that was unable to maintain a prolonged electrophysiological response to a sustained light stimulus (*13*). This phenotype could be mimicked by the application of a non-specific Ca^{2+} blocker, La^{3+} (*13*). The *trp* gene was cloned and shown to encode an integral membrane protein (*34-36*). TRP is believed to be a homotetramer with each subunit composed of six transmembrane domains, and a loop region between the fifth and sixth transmembrane domains that forms the channel pore (Figure 4A) (*37*). At its N-terminus there are three or four ankyrin repeats, each consisting of a 33 amino acid residue motif. Ankyrin repeats may mediate binding of membrane proteins to the cytoskeleton and may also play a role in the channel subunit interactions. There are several sequences motifs at the C-terminus of TRP that may be critical for its regulation (Figure 4A). Immediately

downstream of the sixth transmembrane domain, there is a stretch of 25 amino acids referred to as the TRP domain, which contains six highly conserved amino acids (EWKFAR) called the TRP box (Figure 4A). The TRP box has been suggested to be important for PIP_2 binding (*38*). TRP also contains a putative calmodulin binding domain between residues 682 and 977 (*34, 39*). Downstream of the calmodulin binding motif, there is a PEST region (Figure 4A) that is thought to be important for degradation of TRP by the Ca^{2+}-dependent protease calpain (*38*); however, the exact role of the PEST sequence in TRP is not known because TRP has a very slow turnover rate *in vivo* (*40*). Near the PEST region there is a Pro-rich region containing 27 Lys-Pro repeats that may influence the conformation of the pore domain (*41*). Another stretch of eight amino acids (DKDKKPG/AD) in the C-terminus, which repeats nine times, is likely to mediate protein-protein interactions (*38*). The extreme C-terminus of TRP contains a PDZ-binding domain that is involved in its interaction with INAD (*42*). In *Drosophila*, the TRP Ca^{2+} channel is critical for fly vision and is responsible for more than 90% of photoreceptor depolarization. TRP is 10-fold more abundant than TRPL channels in photoreceptor cells, and does not undergo light-dependent translocation as does TRPL (*43*). The light-dependent translocation of TRPL from rhabdomeres to the cell body is important for light adaptation (*44*). How light leads to activation of TRP is not completely understood. Originally, TRP was thought to be a store-operated channel (SOC), opened by the depletion of intracellular Ca^{2+} stores, *via* activation of the IP_3 receptor. However, null mutations in the sole IP_3 receptor gene of *Drosophila* have no effect on visual signaling (*30*). Recent reports propose that DAG or other lipid mediators, polyunsaturated fatty acids (PUFAs) may gate the TRP channel in *Drosophila*.



B

Subfamily	Worms	Flies	Mice	Humans
TRPC	3	3	7	6[1]
TRPV	5	2	6	6
TRPM	4	1	8	8
TRPA	2	4	1	1
TRPN	1	1	0	0
TRPP[2]	1	1[3]	3	3
TRPML	1	1	3	3
Total	17	13	28	27

Figure 4. *Drosophila* TRP Ca^{2+} channel is the founding member of the TRP channel superfamily. (**A**) Schematic diagrams showing *Drosophila* TRP with distribution of its domains (*top*, see description in text). TRP consists of six transmembrane domains (S1-6), with intracellular N- and C-terminal sequences. Between the fifth and sixth transmembrane domains a loop region contributes to formation of the pore of the channel (*bottom*). It is thought that the *Drosophila* TRP channel is a homotetramer, similar to the voltage-gated K^+ channel. TRP d., TRP domain; CaM b.d., calmodulin binding domain; Pr, Pro rich region; PDZ b.d., PDZ binding domain. (**B**) Table showing the number of TRP channels found in various species. In humans, more than 25 TRP channels have been discovered to date (from Montell, 2005)**.** (**C**) Phylogenetic tree of TRP channels. *Drosophila* TRP is a member of the TRPC family, closely related to TRPC1, 4 and 5 (adapted from Montell, 2005).

Supporting evidence includes the observation that elevated DAG concentrations lead to constitutive activation of TRP in *rdgA* flies that lack DAG kinase (*28*). In addition, direct application of PUFAs reversibly activates the TRP channel in *Drosophila* (*29*). The identity of the secondary messenger that gates the TRP channel remains under further investigation.

INAD

Although the mechanism of TRP opening is not completely understood, TRP is known to associate with INAD, a scaffolding protein that tethers several components necessary for the *Drosophila* visual signaling. It is well established that three proteins, including NORPA, the TRP channel and an eye-specific isoform of protein kinase C (eye-PKC), are constitutively anchored to INAD. INAD imposes appropriate localization of these proteins within the microvilli of rhabdomeres, where visual signaling takes place, thereby assuring the accuracy and speed of phototransduction. INAD mediates these protein-protein interactions through its five PDZ domains. A PDZ domain is a protein-protein interaction domain that is composed of around 90 amino acids with a conserved Gly-Leu-Gly-Phe motif. This motif typically binds to three or four C-terminal residues of a target protein. NORPA interacts with INAD *via* PDZ 1 (*120*), PDZ 3, 4, 5 (*45*), and PDZ 5 (*120*), whereas TRP binds to INAD *via* PDZ 3 (*42*) and PDZ 4 (*135*). Eye-PKC interacts with PDZ 2 in INAD (*54, 136*).

Tsunoda *et al.* (*46*) demonstrated that INAD is responsible for the protein stability of NORPA, eye-PKC and TRP: in $inaD^{1}$ flies lacking INAD, the levels of these proteins are reduced drastically. Interestingly, eye-PKC and NORPA require INAD for their

correct targeting to rhabdomeres (*40*), whereas TRP requires INAD not for targeting, but for its retention within rhabdomeres. In *InaD*^{p215}, a mutant fly in which INAD does not interact with TRP (*42*), TRP undergoes mislocalization and degradation over time (*42, 47*). In addition to protein stability, INAD also confers proper subcellular localization of eye-PKC, NORPA and TRP (*40*). Co-localization of these signaling proteins assures rapid activation and deactivation. It has been proposed that INAD complexes are connected to each other *via* PDZ-PDZ interactions (PDZ 3 or PDZ 4) (*48*), and also that NORPA within these complexes may form homodimers through its C-terminal domain (*49*). These data suggest the existence of a 'signalplex', a higher order signaling network that assures the stoichiometry, speed and specificity of visual signaling (*48*). INAD has also been reported to bind rhodopsin, calmodulin, TRPL and a nonconventional myosin, neither-inactivation-no-afterpotential C (NINAC) (*39, 48, 50*).

A human INAD homologue (hINADL) and two N-terminally truncated isoforms (hINADL$_{\Delta 853}$ and hINADL$_{\Delta 304}$) have been cloned and reported to be expressed in the cerebellum (*51*), yet their retinal expression has not been investigated. The exploration of human INAD expression within RGCs will be of interest, since these vertebrate photoreceptor cells utilize a *Drosophila*-like visual signaling pathway.

Eye-PKC

Once the light stimulus ceases, deactivation of the visual response, and hence inactivation of TRP and TRPL channels, occurs in less than 20 ms (*52*). *Drosophila* eye-PKC is a conventional PKC isoform that is expressed only in the eye. Eye-PKC is critical

for deactivation of visual response as flies lacking eye-PKC (*inaC*^{p209}) exhibit abnormal termination of the light response and defects in light adaptation (*53*).

By immunofluorescence, eye-PKC has been shown to co-localize with INAD and TRP in rhabdomeres (*46, 47, 53*). Using yeast two-hybrid and ligand overlay assays, Adamski *et al*. (1998) demonstrated that eye-PKC interacts with the second PDZ domain of INAD (*54*). The *in vivo* function of eye-PKC depends upon its interaction with INAD, since flies lacking the PDZ-binding motif exhibit prolonged inactivation of the light response, similar to that of *inaC*^{p209} flies, which lack eye-PKC (*54*). To unveil the molecular details responsible for this abnormal phenotype, investigators started to look for substrates of eye-PKC within the INAD complex. Indeed, eye-PKC was shown to phosphorylate both TRP and INAD in *Drosophila* and *Calliphora* (*55-57*). INAD has been suggested to act as an adaptor protein for eye-PKC in a manner similar to the receptor for activated C kinase (RACK) (*55*).

Regulation of *Drosophila* visual transduction

Light initiates a cascade of intracellular events within the *Drosophila* eye that results in opening of TRP and TRPL channels, which results in an increase of Ca^{2+} concentration to about 1 mM in rhabdomeres (*58*). Subsequently, this massive increase in Ca^{2+} concentration leads to depolarization of the photoreceptor cell. It is well known that prolonged elevation of intracellular Ca^{2+} concentrations can lead to cell death. Therefore, levels of intracellular Ca^{2+} are regulated tightly within the photoreceptor cell, not only to maintain cellular homeostasis, but also to fine-tune signal transduction for which temporal resolution is critical. Ca^{2+} is removed from rhabdomeres *via* the Na^{+} / Ca^{2+}

exchanger or the diffusion to the cell body. Within the cell body of the photoreceptor cell, Ca^{2+} is removed from the cytosol by either a Ca^{2+}-ATP-ase that helps sequestrating Ca^{2+} into the ER, or by Ca^{2+}-binding proteins. It has been shown recently that calnexin, in addition to its role in rhodopsin maturation, is a Ca^{2+}-binding protein that has a critical role in regulation of the Ca^{2+} concentration inside the photoreceptor cell (*20*). Calnexin is a transmembrane protein whose N-terminus resides within the ER lumen, whereas its C-terminus is localized in the cytosol. Calnexin contains two Ca^{2+}-binding sites. One of these Ca^{2+}-binding sites is localized within the cytosolic C-terminus, and is thought to prevent Ca^{2+} toxicity and support photoreceptor cell survival (*20*) by buffering the high concentrations of intracellular Ca^{2+}.

It has been shown previously that constitutive activation of TRP leads to retinal degeneration (*59*), possibly through a mechanism similar to that of neurodegenerative excitotoxicity (*60*). Thus, termination of the light response by shutting down TRP is vital not only for temporal resolution of the visual response but also for avoiding Ca^{2+} toxicity from uncontrolled TRP activity. How the visual response is terminated is still under investigation; however, it is likely that it involves multiple mechanisms. Various cellular events appear to contribute to the inactivation of visual signaling, including increased intracellular Ca^{2+} concentrations, the GTP-ase activity of NORPA, and phosphorylation of key protein players.

Ca^{2+}

Ca^{2+} plays a crucial role in deactivation of the visual signaling (*32, 61-63*). Studies on isolated *Drosophila* photoreceptor cells, using fluorescent Ca^{2+}-dyes and

"caged" Ca^{2+} have shown that deactivation of the light response depends on the concentration of extracellular Ca^{2+} (*64*). Following light stimulation, Ca^{2+} enters the photoreceptor cell primarily *via* the TRP channel, and generates localized intracellular Ca^{2+} transients that are about 1 mM (*65*). These Ca^{2+} transients are spatially restricted to rhabdomeres, where the transduction machinery resides. The organization in INAD complexes assures the high temporal resolution of phototransduction. However, how Ca^{2+} mediates the deactivation of the light response is not known. Rapid termination of the visual response may require Ca^{2+} to act on multiple target proteins *via* Ca^{2+}-dependent enzymes such as Ca^{2+}/calmodulin-dependent protein kinase (CaMK) or/and eye-PKC.

The GTP-ase activity of NORPA

Cook *et al.* (2000) investigated *norpA* mutants expressing low levels of PLCβ, and proposed that NORPA accelerates the rate of GTP hydrolysis in Gqα (*66*). Thus, NORPA is suggested to have a dual role, as both an effector and a negative regulator of visual signaling. The mechanism of Gqα inactivation directed by the activation of NORPA by Gqα itself, may assure that Gqα-GTP remains in its activated state until it encounters a NORPA molecule, and that only that particular NORPA molecule will be activated, thereby contributing to maintenance of the stoichiometry of the cascade (*66*).

Phosphorylation of key protein players

Phosphorylation is one of the most common post-translational modifications that regulate signal transduction. One obvious advantage of phosphorylation is the reversibility of the reaction, which permits dynamic regulation of a protein's activity.

This is accomplished by the complementary actions of kinases and phosphatases. In *Drosophila*, several proteins involved in phototransduction have been shown to undergo phosphorylation, such as rhodopsin, arrestins, INAD, and TRP.

Rhodopsin and Arrestins

It is well established that activated rhodopsin is phosphorylated at serine and threonine residues in a light-dependent manner by rhodopsin kinase (*67*). A recent study reported that G-protein coupled receptor kinase 1 is the rhodopsin kinase responsible for phosphorylation of the C-terminal sequence of rhodopsin (*68*). Phosphorylated rhodopsin interacts with arrestin (Arr), which quenches the activity of activated rhodopsin by impeding the direct coupling of rhodopsin and the G-protein (*69*). In *Drosophila* photoreceptors, there are two arrestins: Arr1 and Arr2 (also known as phosrestin 1). Arr1 (39 kDa) has a shorter C-terminus, and is seven-fold less abundant than Arr2, whereas Arr2 (49 kDa) has a long C-terminus, and is the most abundant Arr isoform in the fly eye (*70*). It appears that each Arr has a well-defined function inside the cell. Arr1 binds phosphorylated rhodopsin and mediates the light-induced endocytosis of phosphorylated rhodopsin, thereby attenuating the amplitude of the light response (*70*). In contrast, Arr2 binds to both unphosphorylated and phosphorylated activated rhodopsin molecules, and quenches their signaling. The fact that Arr2 can also bind to the unphosphorylated rhodopsin explains why transgenic flies expressing a modified rhodopsin that lacks phosphorylation sites at its C-terminus displayed an unaltered response to light (*69*). Further work is needed to clarify the mechanistic bases for the two distinct functions of arrestins.

Drosophila arrestins also undergo light-dependent phosphorylation. The first indication of phosphorylation was the observation that these proteins together with an 80 kDa protein, exhibited an anode-shifted migration in two-dimensional gel electrophoresis when isolated from light-treated flies (*67*). Interestingly, phosphorylation of the 80 kDa protein and Arr2 were rapidly reversible in the dark (within 5 minutes for the 80 kDa protein and 1 hour for Arr2) (*67*). Arr2 is known to be phosphorylated at Ser^{366} (*71*) by CaMK II (*72*). However, a modified Arr2 lacking this phosphorylation site is still able to bind rhodopsin, but it is incapable of releasing from the photoreceptor cell membrane once rhodopsin has been converted back to its inactive form (*73*), indicating that phosphorylation regulates the release of Arr2 from membranes. Flies expressing the modified Arr2 lacking the phosphorylation site at Ser^{366} displayed light-dependent retinal degeneration (*73*).

INAD and TRP

As mentioned above, an 80 kDa protein has been known to undergo light-dependent phosphorylation (*67*). However, it was 15 years later when the 80 kDa protein was identified to be the *inaD* gene product, following analysis of tryptic mass fingerprints obtained by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) and Edman degradation (*74*). Based on the MALDI-TOF spectrum, the phosphorylated amino acids of INAD were tentatively assigned. Investigators have suggested that INAD may be the target of multiple kinases, since the putatively assigned phosphorylation sites included Ser, Thr and Tyr residues (*74*).

Evidences supporting eye-PKC phosphorylation of INAD and TRP come from the results of immunocomplex kinase assays (*55*). Experimentally, the INAD complex was immunoprecipitated from head extracts of wild-type or mutant flies lacking specific components of the visual signaling pathway, and subjected to *in vitro* kinase assay. The phosphorylation of both INAD and TRP was found to be dependent on co-purification of eye-PKC, since phosphorylation of INAD and TRP was not detected in INAD complexes from flies lacking eye-PKC. The same results were reported in *Calliphora*, in which the phosphorylation studies were employed using either intact photoreceptor cells followed by isolation of INAD or TRP by immunoprecipitation, or followed by immunocomplex kinase assays (*56, 75*).

Diversity of TRP channel regulation by phosphorylation

As mentioned before, TRP channels are expressed in many excitable and non-excitable cells, and can be subdivided into seven subfamilies (TRPC, TRPV, TRPM, TRPN, TRPA, TRPP, and TRPML) based on amino acid sequence homology (Figures 4B and C) (*37*).

The members of the "canonical" or "classical" TRPC subfamily are nonselective cation channels that are activated through PLC-coupled signaling pathways. For instance, TRPC1, TRPC2, TRPC4 and TRPC5 channels are SOCs that are activated by the release of Ca^{2+} from internal stores, whereas TRPC6 and TRPC7 are activated by DAG (*76*).

The TRPV subfamily comprises channels that are activated by vanilloid compounds such as capsaicin, which is found in hot chili pepper, heat and hypotonic changes. Moreover, TRPV5 and TRPV6 channels are important for vitamin D-stimulated Ca^{2+} uptake in the kidney and small intestine. However, how these two channels are activated is not known (*76*).

The TRPM subfamily derives its name from the founding member, melastatin (TRPM1), a potent tumor suppressor. Three members of this subfamily, TRPM2, TRPM6 and TRPM7, are unique among known ion channels because their C-terminal sequences contain protein kinase domains (*37*). The functional role of these protein kinase domains remains elusive. However, there is evidence that the protein kinase domain of TRPM7 mediates the effect of adenosine 3',5'-monophosphate (cAMP) by increasing channel-dependent current (*76*). TRPM8 is activated by cold and menthol.

The TRPN subfamily includes channels that are mechanically gated, whereas the TRPA subfamily contains ion channels that are either thermo- or mechano-sensitive. The

activation mechanism of TRPP and TRPML channels is not known, however, it is believed that TRPML may play a role in endosomal acidification and hearing, whereas TRPP may be involved in ovarian follicle maturation and differentiation (*37*).

Drosophila TRP shares the closest homology to the TRPC subfamily (*14*). There are five characteristics that TRPC channels have in common with *Drosophila* TRP including: 1) the predicted topology of six transmembrane domains with the typical pore region lying between the fifth and sixth domains, similar to that of the voltage-gated K^+ channel, 2) the absence of charged residues in the fourth transmembrane domain that usually represent the voltage sensor of voltage-gated channels, 3) the presence of three to four ankyrin repeats in the N-terminus, 4) the presence of a proline-rich sequence in the C-terminus for potentially regulating the conformation of the channel pore (*41*), and 5) a highly conserved TRP domain. The TRP domain consists of 25 amino acids, and contains within it the TRP box, Glu-Trp-Lys-Phe-Ala-Arg. The TRP domain has been shown to be important for PIP_2 binding and gating of TRPM8 and TRPV5 channels (*37*). In addition, the TRP domain has been reported to interact with regulatory proteins referred to as immunophilins. For example, FKBP12 binds to *Drosophila* TRP, TRPC3, TRPC6 and TRPC7. Immunophilin FKB59 binds to TRPL, whereas FKBP52 binds to TRPC1, TRPC4 and TRPC5 (*77*). The functional relevance of TRP-immunophilin interactions remains unknown (*77*). In contrast, the scaffolding protein Homer has also been reported to bind the TRP domain of the TRPC1 channel. Homer is known to mediate the interaction between IP_3R and TRPC1, which is vital for TRPC1 gating (*78*).

Mammalian TRP channels have been shown to be regulated by phosphorylation (Table 1). For example, PKC-dependent phosphorylation of TRPC1 has been reported to

activate the channel (*79*), but the phosphorylation site has not been identified. Moreover, PKC has been also shown to activate TRPM4 (*80*) and TRPV1 (*81, 82*), but the molecular details are still obscure. In contrast, phosphorylation of heterologously expressed TRPC3 at Ser^{712} by PKC inhibited channel activity in HEK 293 cells (*83*). Ser^{712} is located just downstream of the TRP domain, however, how phosphorylation affects the activity of TRPC3 remains unknown (*84*). Phosphorylation of TRPC5 at Thr^{972} by PKC has been suggested to contribute to desensitization of the channel, because PKC inhibitors or substitution of Thr^{972} to Ala, prevented desensitization of TRPC5 in HEK 293 cells (*85*). Thr^{972} of TRPC5 is present within the PDZ-interacting domain of TRPC5, and phosphorylation at this site may affect this protein-protein interaction and, consequently, the localization of the channel within the cell (*85*). Phosphorylation of the TRPC6A isoform at Ser^{768} and the TRPC6B isoform at Ser^{714} by PKC inactivates these channels (*86*). In addition, PKC has been proposed to inactivate TRPC4 (*87*), TRPC7 (*88*) and TRPM8 (*89*). Interestingly, TRP channels are subject to phosphorylation by other protein kinases including PKA, PKG, CaMK II and tyrosine kinases (see Table 1), which generally leads to activation of these channels. For example, TRPC3 has also been reported to be phosphorylated by PKG (*90*) and Src family tyrosine kinases (STKs) (*91*). Phosphorylation of TRPC4 at Tyr^{959} and Tyr^{972} by a member of the STK family, Fyn, led to activation of the channel (*84*). Moreover, tyrosine phosphorylation enhanced the association between TRPC4 and the scaffolding protein NHERF (Na^+/ H^+ exchange regulatory factor) that increases the membrane insertion of this channel and consequently, its activity (*84*). In addition, STKs (Fyn) phosphorylate TRPC6 (*92*), TRPM7 (*93*) and TRPV4 (by Lyn) (*94*), leading to activation of these channels. CaMKII phosphorylates

and activates TRPC6 (*88*) and TRPV1 (*95*), whereas PKA phosphorylates and activates TRPV1 (*82, 96*).

The role of macromolecular complexes in regulating PKC substrate specificity

Classical PKCs are serine/threonine protein kinases that transduce cellular signals derived from lipid hydrolysis and Ca^{2+}. PKCs have been implicated in a multitude of physiological and pathological processes such as vision (*12*), diabetic retinopathy (*97*), learning and memory (*98*), stroke-induced damage (*99*), hypertension (*100*) and cancer (*101*). At the molecular level, PKC contains a regulatory and a catalytic domain. The regulatory domain consists of an autoinhibitory region, the pseudosubstrate, and two subdomains, C1 and C2, that bind DAG and phorbol esters, and acidic phospholipids and Ca^{2+}, respectively (*102*). To date 12 different PKC isozymes have been identified in vertebrates. They are classified into three groups according to the structure of regulatory domains that, consequently, determines sensitivity to different cofactors. Conventional PKCs (α, βI, βII and γ) respond to both Ca^{2+} and DAG, whereas novel PKCs (δ, ε, η/L and θ) respond only to DAG. In contrast, atypical PKCs (ζ and ι/λ) are insensitive to both Ca^{2+} and DAG (*101*).

The subcellular localization of PKC is important for its substrate specificity. The protein substrate that PKC interacts with is called substrate that interacts with C-kinase (*101*). Sometimes, PKC targets its substrates *via* a scaffold or adaptor protein. The adaptor protein may act either as a receptor for inactivated C-kinase (RICK) or a receptor for activated C-kinase (RACK) (*101*). Although there are many studies addressing phosphorylation of various ion channels by PKC (see Table 2), there have been only a

Table 1. TRP channels known to undergo phosphorylation.

Channel	Kinase	Functional effect	Phosphorylation site(s)	References
TRPC1	PKC	activation	?	(*55*)
TRPC3	PKC	inactivation	Ser^{712}	(*56*)
	PKG	inactivation	Thr^{11}, Ser^{263}	(*57*)
	Src	activation	Tyr^{226}	(*86, 93*)
TRPC4	PKC	inactivation	?	(*79*)
	Fyn	activation	Tyr^{959}, Tyr^{972}	(*76*)
TRPC5	PKC	inactivation	Thr^{972}	(*77, 79*)
TRPC6	Fyn	activation	?	(*87*)
	PKC	inactivation	Ser^{768} (**TRPC6A**), Ser^{714}(**TRPC6B**)	(*78*)
	CaMK II	activation	?	(*80*)
	PKA	?	?	(*91*)
	PKG	?	?	(*91*)
TRPC7	PKC	inactivation	?	(*80*)
TRPM4	PKC	activation	?	(*82*)
TRPM7	Src	activation	?	(*88*)
TRPM8	PKC	inactivation	?	(*81*)
TRPV1	PKA	activation	Ser^{116}, Thr^{370}	(*84, 92*)
	PKC	activation	?	(*83, 84*)
	CaMKII	activation	?	(*90*)
TRPV4	Lyn	activation	Tyr^{253}	(*89*)

few studies exploring the involvement of the adaptor proteins in achieving the rapid regulation of ion channels by PKC. For instance, Hoshi *et al*. (2003) reported that the action of PKC on KCNQ is regulated by scaffolding protein A-kinase anchoring protein 150, which binds both PKC and KCNQ, and supports PKC-mediated inhibition of the channel (*103, 104*).

In addition, the PKCζ interacting protein known as ZIP, has been demonstrated to interact with PKCζ and the Kvβ2 subunits of the K^+ channel in the hippocampus and Purkinje cells, to attain specificity of the PKCζ-targeted phosphorylation of the K^+ channel (*105*). Another member of the ZIP protein family, ZIP3, which is expressed in the mammalian retina, has been found to form a complex between PKCζ and the ρ3 subunit of the GABAc receptor, a ligand-gated Cl^- channel that is important for vision (*106*). Croci *et al.* (2003) speculated that the long intracellular loop between transmembrane regions 3 and 4 of the GABAc receptor may be the target of PKC phosphorylation to mediate inhibition of the channel, since this region of the channel contains PKC consensus phosphorylation sites (*107*).

In addition, CFTR is a Cl^- channel whose improper functioning is responsible for the pathology of cystic fibrosis (*108*). CFTR is regulated by PKCε *via* two proteins, RACK1 and NHERF1. Liedtke *et al.* (2002) found that RACK1 interacts with PKCε and NHERF1. NHERF1 also interacts with CFTR, and therefore, it is believed that RACK1 and NHERF1 serve as scaffold proteins to anchor PKCε in close proximity to the CFTR channel (*109*).

PKCε has been demonstrated to bind to a PKC binding protein named enigma homolog (ENH), which also interacts with voltage-gated N-type Ca^{2+} channels in

hippocampal neurons. The PKCε-ENH-calcium channel macromolecular complex facilitates the action of PKCε on the channel (*110*).

The data presented above highlight the importance of macromolecular complexes in attaining the specificity and efficiency of cellular signaling. In these complexes, PKC is targeted to ion channels with the help of adaptor proteins, which promote close proximity of PKC to its channel substrates.

Table 2. PKC mediates the regulation of various ion channels.

Ion Channel	Function	PKC-mediated effect	Phosphorylation site(s)	References
Na^+ Channels				
Cerebral voltage- gated Na^+ channel	- initiation and propagation of action potentials -synaptic plasticity	decreased amplitude	Ser^{1506} of α-subunit	(*111*, *112*)
Na(v)1.9	-peripheral nociception	activation	?	(*113*)
Skeletal muscle voltage-gated Na^+ channel	-skeletal muscle contraction	inactivation	?	(*114*)
Calcium Channels				
L-type Calcium channel	-vasoconstriction	activation	?	(*115*, *116*)
Cav1.1	-skeletal muscle contraction	inactivation	?	(*117*)
Cav1.2	-cardiac response to hormonal regulation	activation	Ser^{1928}	(*118*)
K^+ Channels				
TREK-1 (KCNK2, K2P2.1)	-maintaining the membrane potential in neurons (background K^+ current)	inactivation	Ser^{333}	(*119*)
M- type (KCNQ2)	-synaptic plasticity - negative control over neuronal excitability	inactivation	Ser^{534}, Ser^{541}	(*104*, *103*)
Kir3	-maintaining the membrane potential in neurons -inhibit hormone release -vagal-mediated bradicardia	inactivation	Ser^{503} (K(v) 3.1b)	(*121*, *122*)
K(v)4.2/4.3 channels	- electric remodeling by cell volume changes (*e.g.* cell swelling or hypertrophy)	inactivation	?	(*123*)
Other Channels				
AMPAR	- fast excitatory neurotransmission	AMPAR trafficking	Ser^{880}(GluR2)	(*124-126*)
GluR2δ	-cerebellar synaptic plasticity	?	Ser^{945}	(*127*)
NMDAR	- synaptic plasticity - learning and memory	desensitization	S^{890} and S^{896} (NR1) S^{900}, S^{929} and S^{1416} (N2A) S^{1303} and S^{1323}(NR2B)	(*128-132*)
CTRL	-mucus and sweat	activation	Ser^{660}, Ser^{686}, Ser^{700}, Ser^{790}	(*108*)

Summary

The above review regarding regulation of ion channels by PKC illustrates the critical role of PKC-mediated phosphorylation in a large number of cellular events. PKC is important to regulate visual signaling in *Drosophila*, a PLCβ-coupled signaling cascade in which the TRP Ca^{2+} channel mediates the light-dependent depolarization of photoreceptor cells. *Drosophila* visual signaling is of particular interest not only because it is one of the fastest known G-protein coupled signaling pathways, but also because working with *Drosophila* provides ease of handling and phenotype assessment, genetic maneuverability, a database of existing mutants, and a sequenced genome. Uncovering the details concerning the regulation of TRP by eye-PKC for insight into deactivation of *Drosophila* visual response and light adaptation, will provide a better understanding of the regulation of ion channels, particularly, TRPs, by PKC-dependent phosphorylation.

Specific Aims

The work presented here seeks to determine the molecular details of the regulation of *Drosophila* visual signaling by eye-PKC. More specifically, I was interested in investigating how PKC regulates the activity of the TRP Ca^{2+} channel. Eye-PKC is critical for modulating visual signaling, and has been shown to phosphorylate TRP *in vitro*. The specific aims for my project were: 1) to identify the eye-PKC target region of TRP by *in vitro* phosphorylation assays; 2) to map the *in vivo* eye-PKC phosphorylation site(s) in TRP using mass spectrometry; and 3) to investigate how phosphorylation modulates the function of TRP by generating transgenic flies lacking the phosphorylation site(s), and assessing their visual phenotype.

CHAPTER II

SCAFFOLDING PROTEIN INAD REGULATES DEACTIVATION OF VISION BY PROMOTING PHOSPHORYLATION OF TRP BY EYE-PKC IN *DROSOPHILA*

Introduction

Drosophila visual transduction is a G-protein-coupled signaling pathway that provides a model system for understanding the molecular basis of signal transduction in the vertebrate nervous systems. *Drosophila* visual signaling is initiated upon activation of rhodopsin by light. Activated rhodopsin, *via* a Gq heterotrimeric protein, stimulates phospholipase Cβ (PLCβ) named NORPA (*9*). NORPA hydrolyzes phosphatidylinositol-4, 5-bisphosphate (PIP_2) to inositol 1, 4, 5 trisphosphate (IP_3) and 1, 2 diacylglycerol (DAG), which leads to opening of the TRP Ca^{2+} and TRP-like channels, and depolarization of photoreceptors (*26, 27*). The key second messenger that activates the TRP Ca^{2+} channel is thought to be either DAG or its lipid metabolites (*28, 29*), whereas IP_3 does not appear to play a role (*10, 30*). DAG may have a dual function, because it also activates the eye-specific PKC (eye-PKC) essential for deactivation of the light response (*31, 32*).

Drosophila visual signaling is one of the fastest G-protein-coupled transduction cascades (*65*). The fast kinetics of vision is partly due to the formation of a macromolecular complex containing TRP Ca^{2+} channel (*42, 135*), NORPA (*46, 54, 136*) and eye-PKC (*46, 54, 136*). This complex is organized by INAD (*137*), a scaffolding protein with five PDZ domains. INAD regulates the subcellular localization and stability of these three proteins. Flies lacking INAD exhibit a profound reduction of the light

response (*46*).

To gain a better understanding of how the INAD complex modulates the kinetics of vision, we and others have shown that the INAD-TRP interaction is required for normal deactivation of the light response because a loss of the interaction leads to slow deactivation in *InaD*^{p215} flies (*137, 138*), In addition, the INAD-eye-PKC interaction is essential for the *in vivo* activity of eye-PKC as expression of modified eye-PKC that does not interact with INAD, fails to rescue *inaC*^{p209} flies lacking eye-PKC (*54*). Indeed, two proteins in the complex, INAD and TRP, were found to be phosphorylated *in vitro* by eye-PKC (*56, 57*).

Here we report the identification and functional characterization of an eye-PKC phosphorylation site in TRP. We show that TRP is phosphorylated at Ser^{982} by eye-PKC and this phosphorylation depends on INAD *in vitro*. By differential mass spectrometry (MS), we confirm that Ser^{982} of TRP is phosphorylated *in vivo* by eye-PKC. Moreover, we demonstrate that transgenic flies lacking this phosphorylation site display a slow deactivation phenotype similar to that of *InaD*^{p215}. Our results indicate that INAD is critical for deactivation of visual signaling by positioning eye-PKC in close proximity to TRP, in order to facilitate its phosphorylation at Ser^{982}.

Materials and Methods

Preparation of fly head extracts. Approximately 100 µl young wild-type, *inaD*^{1} or *InaD*^{p215} fly heads were homogenized with 1 ml of extraction buffer or EB (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100 and a mixture of protease inhibitors). Head

homogenates were incubated at 4 °C with constant agitation for 1 h. The mixture was then centrifuged for 10 min (12,000 X *g*), and the supernatant was used for the *in vitro* complex-dependent kinase assay. Protein concentrations were determined by BCA (Pierce).

***In vitro* complex-dependent kinase assay.** This assay consists of a GST pull-down followed by an *in vitro* kinase assay. GST fusion proteins were immobilized to glutathione-agarose beads and incubated with 500 μl fly head extract (total protein concentration 3.5-5 μg/ml) for 1 h at 4°C. After incubation, GST fusion proteins and associated proteins were recovered by centrifugation and washed three times with EB. For the kinase assay, the GST fusion protein mixture was washed once with kinase reaction buffer or RB (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.1 mM DTT, 0.4 mM EGTA, 0.7 mM $CaCl_2$) and incubated at 30^{o}C with 50 μl RB containing phorbol myristate acetate (PMA, 1 μM), 3 μCi of carrier-free [γ-^{32}P]-ATP in the presence of 100 μM cold ATP. 2X SDS/PAGE loading buffer was added to terminate the kinase reactions. Samples were then subjected to SDS/PAGE (6% or 10%) followed by Western blotting or Coomassie Blue staining. Dried and stained gels were subject to autoradiography or PhosphorImager analysis to quantify phosphorylation of fusion proteins.

LC-MS analysis. LC-MS was performed by the Proteomics Laboratory in the Vanderbilt Mass Spectrometry Research Center. About 14 pmoles of TRP were excised from SDS/PAGE gels for in-gel digestion with either trypsin or chymotrypsin (*152*). The

resulting peptides were separated by reverse phase high pressure liquid chromatography that is coupled directly with automatic tandem MS (LC-MS) using a ThermoFinnigan LTQ ion trap mass spectrometer equipped with a Thermo MicroAS autosampler and Thermo Surveyor HPLC pump, Nanospray source, and Xcalibur 1.4 instrument control. MS/MS scans were acquired using an isolation width of 2 m/z, an activation time of 30 ms, and activation Q of 0.250 and 30% normalized collision energy using 1 microscan and ion time of 100 for each scan. The mass spectrometer was tuned prior to analysis using the synthetic peptide TpepK (AVAGKAGAR). Typical tune parameters were as follows: spray voltage of between 1.8 KV, a capillary temperature of 150ºC, a capillary voltage of 50V and tube lens 100V. Initial analysis was performed using data-dependent scanning in which one full MS spectra, using a full mass range of 400-2000 amu, were followed by 3 MS/MS spectra. Incorporated into the method was a data-dependent scan for the neutral loss of phosphoric acid or phosphate (-98, -80), such that if these masses were found, an MS/MS/MS of the neutral loss ion was performed. Peptides were identified using a cluster compatible version SEQUEST algorithm (*140*), using a *Drosophila* subset of proteins from the non-redundant database from NCBI or Uniref100. Sequest searches are done on a high speed, multiprocessor Linux cluster in the Advanced Computing Center for Research. In addition to using the SEQUEST algorithm to search for phosphorylation on serines or threonines, the data were also analyzed using the Pmod algorithm (*162*). All possible modified peptides were verified by manual inspection of the spectra.

P-element-mediated germ-line transformation. Wild-type and modified *trp* cDNA were subcloned into a modified pCaSpeR 4 vector (*133*) that contains *Drosophila hsp70* promoter without the *hsp70* 3' trailer region. The P-element construct and a transposase plasmid ("wings-clipped") were injected into *y [1] w[67c23]* embryos (CBRC transgenic *Drosophila* Core, Massachusetts General Hospital / Harvard Medical School). Flies with the transgene integrated into the second or third chromosome were selected and made homozygote in the trp^{p301} background for further analysis.

Electroretinogram recordings (ERG). ERG recordings were carried out using red-eye young flies (1-3 days old) that were reared in a 12/12 hr light/dark cycle. The flies were anesthetized by carbon dioxide and immobilized using non-drying modeling clay. Glass electrodes were filled with physiological saline (0.7% NaCl). White light stimulation (light intensity, 4.45 mW) was delivered by a fiber optic light source (Oriel) and attenuated using absorptive nd filters (Newport, Irvine, CA). Signals were amplified by means of a WPI Dam 50 differential amplifier (World Precision Instruments, Sarasota, FL), displayed on an oscilloscope. Data were digitalized and analyzed using AxonScope 9.0 software (Axon Instruments, Sunnyvale, CA).

Statistical analysis. All bar graph data were analyzed with GraphPad Prism 4.0 software (San Diego, CA) one-way analysis of variance. Data represent the means ± S.E.M., unless otherwise noted, from several independent experiments.

Results

The C- terminal tail of TRP contains PKC phosphorylation sites

To investigate the regulation of TRP by eye-PKC, we first identified potential eye-PKC phosphorylation sites in TRP. TRP consists of six transmembrane domains with both N- and C-termini localized intracellularly. By NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) and Prosite (http://www.expasy.ch/prosite/) software using the PKC consensus sequence motif (S/T)-X-(R/K), we found 16 putative phosphorylation sites in TRP with 14 present within the C-terminal sequence (Figure 5A). Since the C-terminal tail of TRP has been implicated in gating and regulation of the channel, phosphorylation of this region may serve to switch on/off the channel activity. To investigate whether any of the putative PKC sites are *bona fide* PKC phosphorylation sites, we generated GST fusion proteins containing different intracellular regions of TRP and subjected them to *in vitro* kinase assays. As positive and negative controls we used a fusion protein containing full length INAD and GST alone, respectively. We first determined whether a recombinant PKCα could phosphorylate these fusion proteins because both PKCα and eye-PKC belong to the conventional PKC family. Indeed, we found that $TRP^{906-1275}$ containing the last 370 residues of TRP including the six putative PKC sites became phosphorylated by PKCα (Figure 5B), whereas TRP^{1-367} which contains two PKC sites did not (data not shown). Sequences spanning $TRP^{657-905}$ failed to produce stable fusion proteins in *E. coli* and therefore were not tested. The stoichiometry of $TRP^{906-1275}$ phosphorylation by PKCα was approximately 0.55 moles phosphate/moles fusion protein.

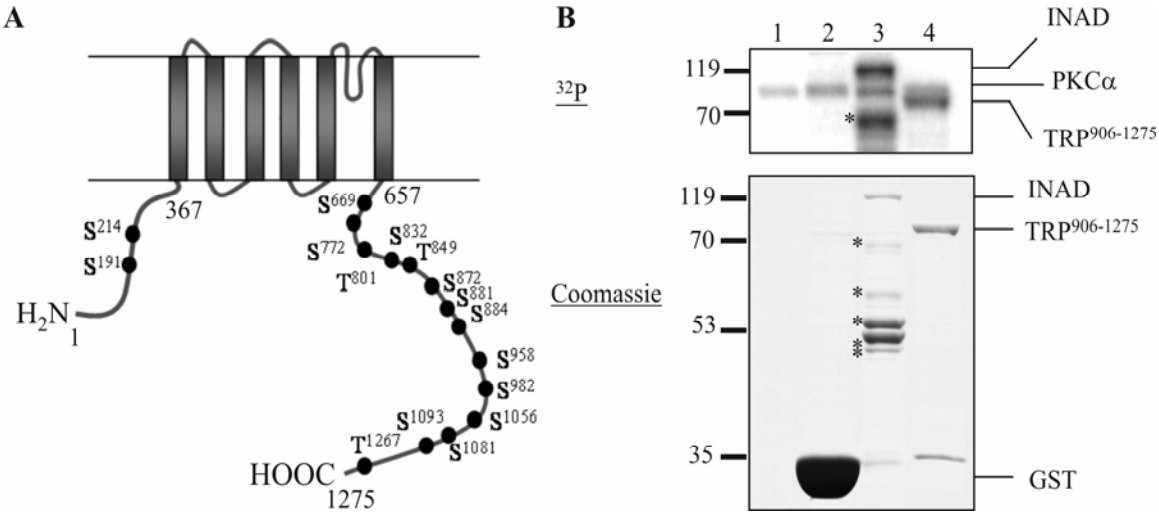


Figure 5. The C-terminal tail of TRP is phosphorylated by recombinant PKCα.
(**A**) Distribution of the 16 putative PKC phosphorylation sites in TRP. (**B**) Phosphorylation of $TRP^{906-1275}$ and INAD by PKCα. *Lane 1*, no substrate; *lane 2*, GST; *lane 3,* INAD; *lane 4,* $TRP^{906-1275}$. *Asterisks* indicate INAD degradation products. The protein standards (kDa) are denoted on the left.

These findings indicated that $TRP^{906-1275}$ contains one PKC phosphorylation site.

Phosphorylation of $TRP^{906-1275}$ by eye-PKC is dependent on INAD *in vitro*

Next we investigated if $TRP^{906-1275}$ can be phosphorylated by eye-PKC. In *Drosophila* photoreceptors, eye-PKC and TRP form a macromolecular complex by tethering to INAD. To obtain eye-PKC, immobilized GST fusion proteins containing $TRP^{906-1275}$ were incubated with wild-type fly head extracts in order to compete with endogenous TRP for retrieval of the INAD complex, including eye-PKC. The resulting complex was recovered by centrifugation and used for *in vitro* kinase assays. We found that $TRP^{906-1275}$ pulled down INAD and eye-PKC, and became phosphorylated upon the addition of a PKC activator, PMA, by this complex-dependent kinase assay (Figure 6A, *lane 2*). To demonstrate that the observed phosphorylation of $TRP^{906-1275}$ is dependent on INAD, we used fly extracts prepared from $inaD^{1}$ and $InaD^{p215}$. $inaD^{1}$ is a loss-of-function allele of *inaD* (*46*), whereas $InaD^{p215}$ expresses a modified protein resulting in a loss of the TRP-INAD association (*42*). As shown in Figure 6A (*lanes 3-6*), both extracts failed to support $TRP^{906-1275}$ phosphorylation by the complex-dependent kinase assay. In both cases, phosphorylation was diminished because $TRP^{906-1275}$ was unable to isolate INAD and consequently, eye-PKC, from these two extracts (Figure 6A**,** *middle* and *bottom*).

To further support the role of INAD in directing eye-PKC to TRP, we investigated phosphorylation of a modified $TRP^{906-1275}$ containing an Asp substitution at Val^{1266}, which has been previously shown to disrupt the interaction between TRP and INAD (*40, 42*). We found that phosphorylation of $TRP^{906-1275, V1266D}$ was greatly reduced (Figure 6B and C), because this modified TRP failed to recruit INAD and consequently,

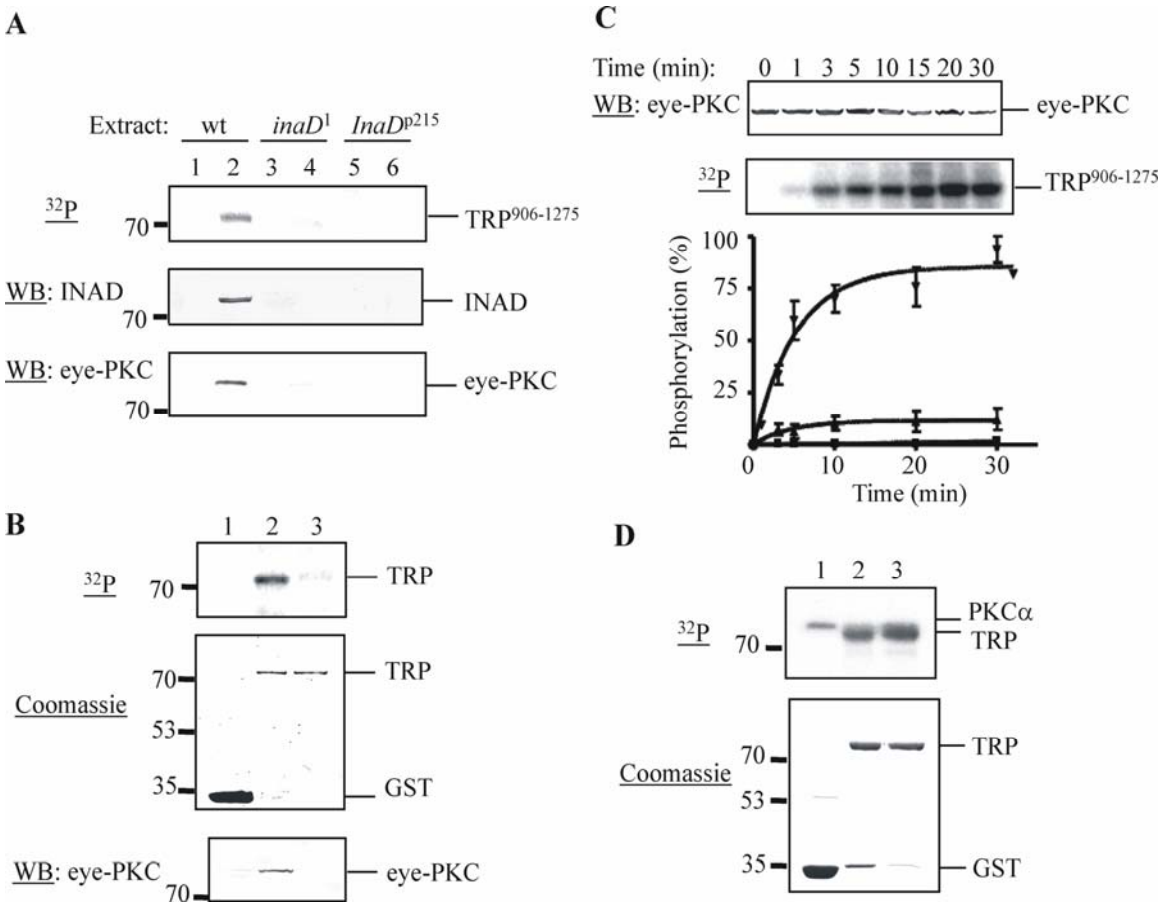


Figure 6. TRP is phosphorylated *in vitro* by eye-PKC in a complex-dependent manner. (**A**) Phosphorylation of $TRP^{906\text{-}1275}$ using wild-type (wt), *inaD*^{1} and *InaD*^{p215} fly head extracts. Recovery of INAD (*middle*) and eye-PKC (*bottom*) was detected by Western blotting. *Lanes 1, 3* and *5*, GST; *lanes 2, 4* and *6*, $TRP^{906\text{-}1275}$. (**B**) Reduced phosphorylation of a modified $TRP^{906\text{-}1275}$ containing an Asp substitution at Val^{1266} by the complex-dependent kinase assay (*top*). The corresponding Coomassie stained SDS-PAGE gel is shown in the *middle*. Recovery of eye-PKC was detected by Western blotting (*bottom*). *Lane 1*, GST; *lane 2*, $TRP^{906\text{-}1275}$; *lane 3*, $TRP^{906\text{-}1275,\ V1266D}$. (**C**) Time-course of the TRP phosphorylation using wild-type fly head extracts. Recovery of eye-PKC by wild-type TRP was detected by Western blotting (*top*). GST (■); $TRP^{906\text{-}1275}$ (▼); $TRP^{906\text{-}1275,\ V1266D}$ (▲). All quantifications were performed as described in *Materials and Methods* (n = 3). (**D**) $TRP^{906\text{-}1275,\ V1266D}$ remains a substrate for recombinant PKCα (*top*). The corresponding Coomassie stained SDS-PAGE gel is shown at the *bottom*. *Lane 1*, GST; *lane 2*, $TRP^{906\text{-}1275}$; *lane 3*, $TRP^{906\text{-}1275,\ V1266D}$.

eye-PKC (Figure 6B, *bottom*). Importantly, this modified TRP remained an excellent substrate for recombinant human PKCα (Figure 6D). Together, these results indicate that the phosphorylation of $TRP^{906-1275}$ by endogenous eye-PKC *in vitro* is dependent on the interaction between TRP and INAD.

To demonstrate that eye-PKC is involved in phosphorylation of $TRP^{906-1275}$, we show that this phosphorylation is abolished in the presence of a specific conventional PKC inhibitor Go6976 (5 μM, Figure 7A). To further confirm that eye-PKC is responsible for the observed phosphorylation of $TRP^{906-1275}$, we carried out the complex-dependent kinase assay using extracts from $inaC^{p209}$ (*139*) that lacks endogenous eye-PKC (*31*). As expected, phosphorylation of $TRP^{906-1275}$ and full-length INAD was greatly reduced, by 82 and 86%, respectively (Figure 7B, $TRP^{906-1275}$, 17.77 ± 2.5 %, INAD, 13.81 ± 4.13 %, n = 3, mean ± S.E.M.). The absence of phosphorylation is due to a lack of eye-PKC recovery when $inaC^{p209}$ extracts were used (Figure 7B, *middle*). These findings indicate that eye-PKC is indeed responsible for phosphorylation of $TRP^{906-1275}$.

TRP is phosphorylated at Ser^{982} *in vitro*

To investigate which of the six putative PKC sites in $TRP^{906-1275}$ is phosphorylated by eye-PKC, we examined phosphorylation of two shorter TRP fusion proteins that contain one ($TRP^{1157-1275}$) or four ($TRP^{1030-1275}$) predicted PKC sites. We found that $TRP^{1030-1275}$ and $TRP^{1157-1275}$ displayed a drastic reduction of phosphorylation by 78 and 91%, respectively (Figures 8A, $TRP^{1030-1275}$, 22.09 ± 7.78 %, $TRP^{1157-1275}$, 8.33 ± 5.43 %, n = 3, mean ± S.E.M.), by eye-PKC (Figure 8B). Interestingly, these two fusion proteins were also not phosphorylated by recombinant PKCα (Figure 8C).

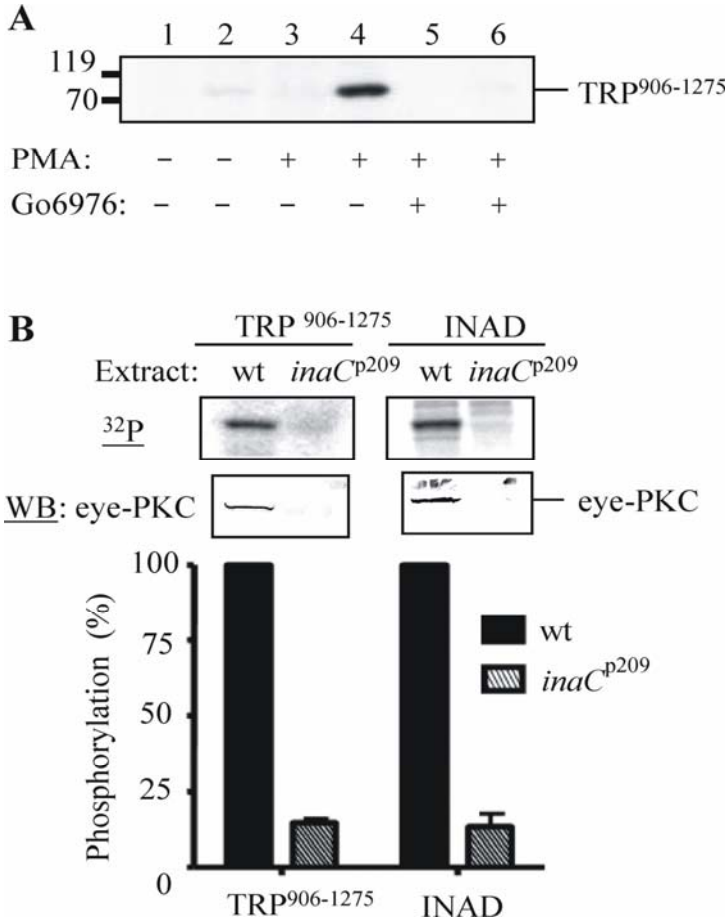


Figure 7. TRP$^{906\text{-}1275}$ is phosphorylated *in vitro* by eye-PKC. (**A**) Phosphorylation of TRP$^{906\text{-}1275}$ was eliminated by a specific conventional PKC inhibitor, Go6976. *Lanes 1*, *3* and *5*, GST; *lanes 2*, *4* and *6*, TRP$^{906\text{-}1275}$. (**B**) *inaC*^{p209} extracts failed to promote the complex-dependent phosphorylation of TRP and INAD. The incorporated radioactivity was normalized to protein levels obtained by densitometry (Odyssey analysis software) and the relative phosphorylation was presented as mean ± S.E.M. (n = 3).

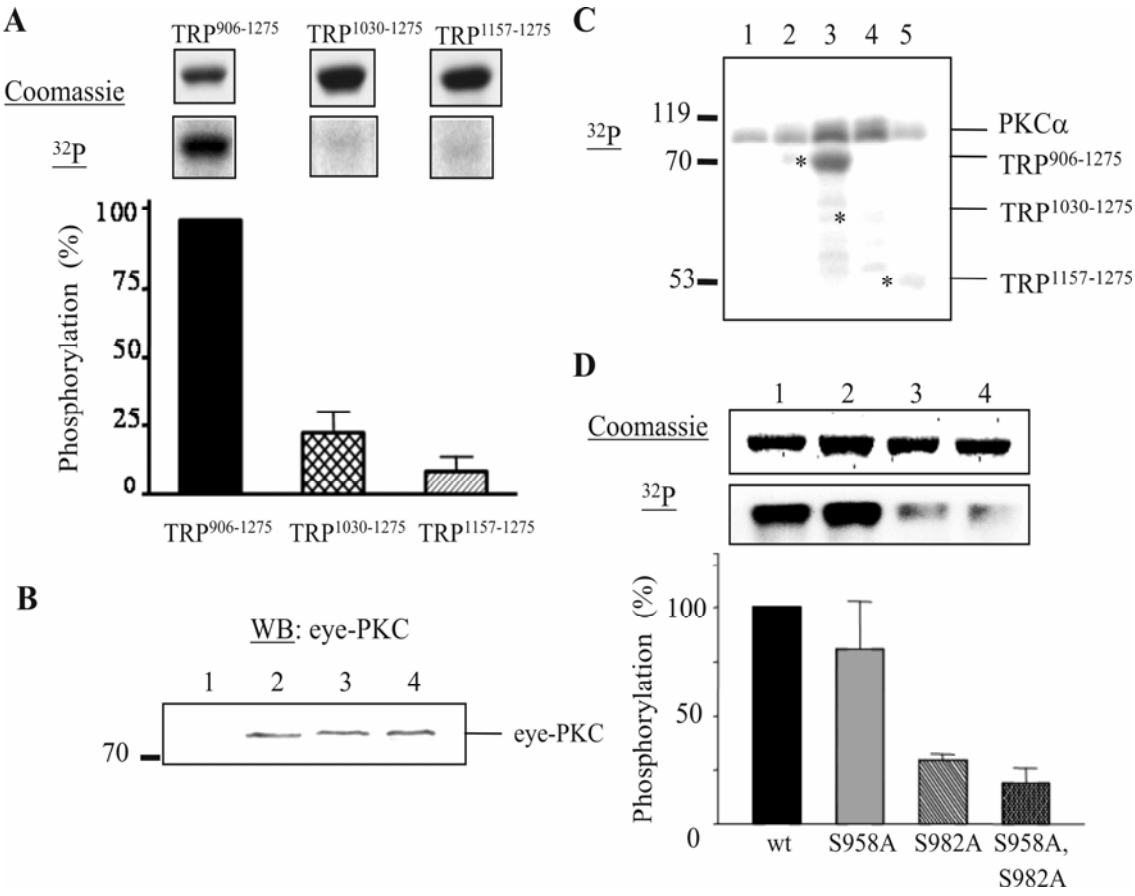


Figure 8. Mapping the PKC phosphorylation site in TRP$^{906\text{-}1275}$. (**A**) The complex-dependent phosphorylation of three TRP fusion proteins: TRP$^{906\text{-}1275}$, TRP$^{1030\text{-}1275}$ and TRP$^{1157\text{-}1275}$. One relevant autoradiography and its corresponding Coomassie stained proteins are shown. (**B**) Eye-PKC is recovered by all three TRP fusion proteins. *Lane 1*, GST; *lane 2*, TRP$^{906\text{-}1275}$; *lane 3*, TRP$^{1030\text{-}1275}$; *lane 4*, TRP$^{1157\text{-}1275}$. (**C**) Phosphorylation of TRP fusion proteins by PKCα. *Lane 1*, no substrate; *lane 2,* GST; *lane 3,* TRP$^{906\text{-}1275}$; *lane 4*, TRP$^{1030\text{-}1275}$; *lane 5,* TRP$^{1157\text{-}1275}$. *Asterisks* denote the location of each TRP fusion protein. (**D**) TRP is phosphorylated *in vitro* at Ser^{982} by PKCα. *Lane 1*, TRP$^{906\text{-}1275}$, *lane 2*, TRP$^{906\text{-}1275,\ S958A}$, *lane 3*, TRP$^{906\text{-}1275,\ S982A}$, *lane 4*, TRP$^{906\text{-}1275,\ S958A,\ S982A}$. All quantifications were done as described before (n = 3). All data are presented as mean ± S.E.M.

These data indicate that Ser^{958} or Ser^{982}, the two sites present in $TRP^{906-1275}$ but not in $TRP^{1030-1275}$, are the potential phosphorylation sites for both eye-PKC and recombinant PKCα.

To investigate whether Ser^{958} or Ser^{982} is indeed the primary PKC phosphorylation site, we generated a modified $TRP^{906-1275}$ containing Ala substitutions at these two sites and performed *in vitro* kinase assays. We found that Ala substitution of Ser^{982} resulted in a marked decrease of phosphorylation by PKCα, whereas a similar substitution at Ser^{958} did not (Figure 8D, $TRP^{906-1275}$, 100 %, $TRP^{906-1275, S958A}$, 80.47 ± 22.24 %, $TRP^{906-1275, S982A}$, 29.46 ± 2.84 %, n = 3, mean ± S.E.M.). Consistently, fusion proteins containing both mutations (S958A and S982A) also exhibited a drastically reduced phosphorylation (Figure 8D, $TRP^{906-1275, S958A, S982A}$, 18.88 ± 6.93 %, n =3, mean ± S.E.M.).

To further confirm that Ser^{982} of TRP is a PKC phosphorylation site, we obtained a synthetic peptide, ALRAS^{982}VKNVD, spanning Ser^{982} of TRP, and used it for *in vitro* kinase assays. This oligopeptide was a substrate of recombinant human PKCα with a *Km* of 263.1 μM and a *Vmax* of 17.35 pmol/min. Taken together, these data indicate that Ser^{982} represents a major *in vitro* PKC phosphorylation site in $TRP^{906-1275}$.

TRP is phosphorylated *in vivo* at Ser^{982} by eye-PKC

Once we established that Ser^{982} of TRP was phosphorylated *in vitro* by eye-PKC, we investigated if Ser^{982} is phosphorylated *in vivo* by eye-PKC using liquid chromatography-MS (LC-MS) analysis. First, we isolated the INAD complexes from light-adapted wild-type flies *via* immunoprecipitation, using anti-INAD antibodies.

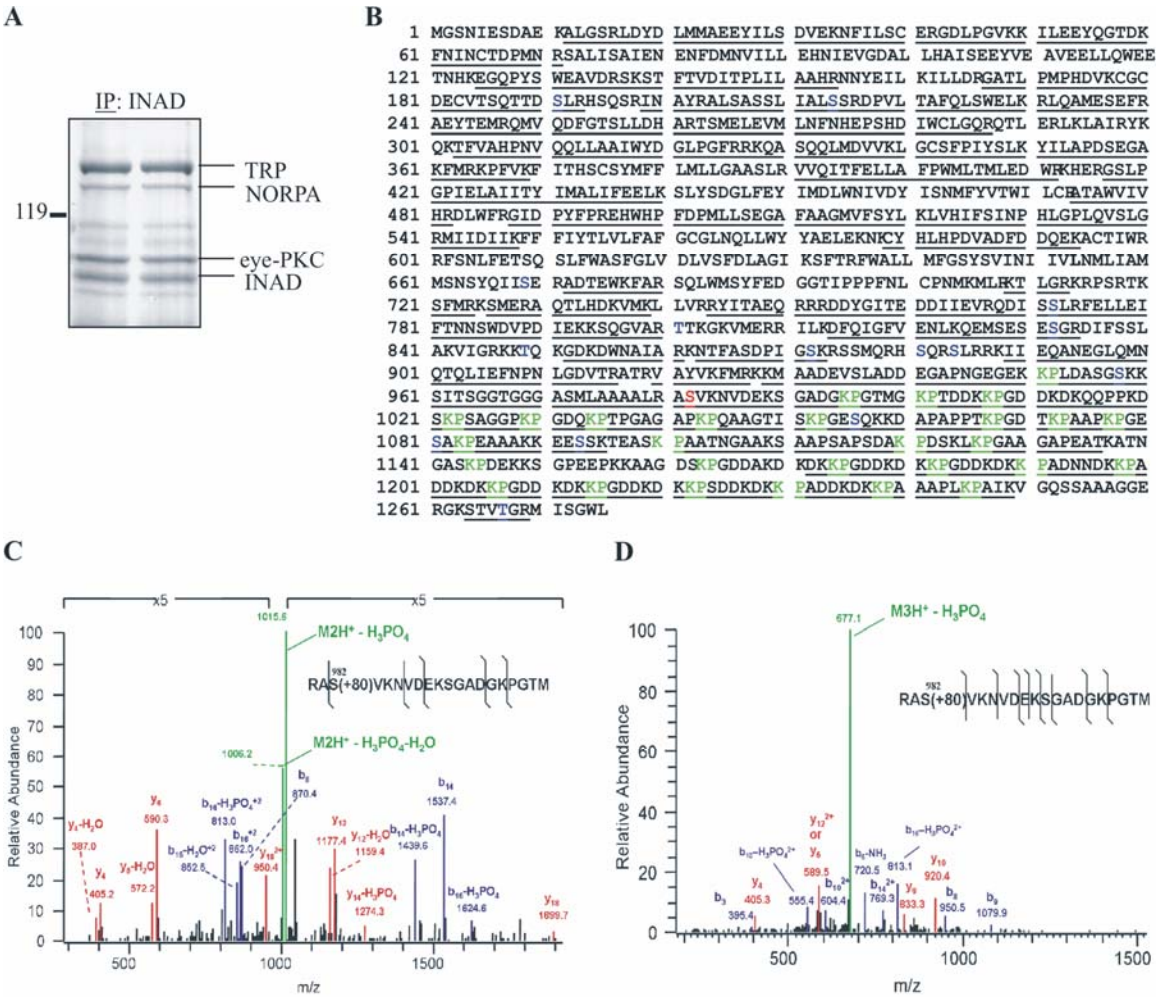


Figure 9. TRP is phosphorylated *in vivo* at Ser^{982} as revealed by LC-MS analysis. (**A**) Colloidal Blue staining of a gel from which TRP was recovered following immunoprecipitation. (**B**) The sequence coverage of TRP was approximately 70%, including 11 putative PKC sites. *Solid lines* denote tryptic and chymotryptic peptides identified by MS. Putative PKC phosphorylation sites are coded *blue*, Ser^{982} is coded *red*, and Lys-Pro repeats are coded *green*. (**C**) Low energy CID spectrum of the doubly charged phosphopeptide Arg^{980}-Met^{999}. The spectrum was zoomed in 5-fold due to a very prominent neutral loss of ion. (**D**) A low energy CID spectrum of the triply charged phosphopeptide spanning Arg^{980}-Met^{999}. The insets show the sequence of the phosphopeptide; *black lines* denote the identified cleavages. Fragment ions are labeled according to the accepted nomenclature. *b*-ions are coded *blue*, *y*-ions, *red*, and precursor ions, *green*. Spectra from the MS/MS/MS analysis of the neutral loss of phosphoric acid ions confirmed the sequence and the site of phosphorylation (data not shown).

The proteins in the INAD complexes were separated by SDS-PAGE and visualized by staining with Colloidal Blue. The 145 kDa protein band corresponding to TRP was excised (Figure 9A), digested "in-gel" with trypsin or chymotrypsin, and the resulting peptide mixture was subjected to LC-MS analysis. Peptide fragments were analyzed and identified by a cluster compatible version SEQUEST algorithm (*140*), using a subset of *Drosophila* proteins from the NCBI database. We obtained approximately 70% amino acid coverage of TRP (Figure 9B) including sequences spanning Ser^{982}. We also used Collision induced dissociation (CID), which fragments peptides such that the fragmentation pattern can be used to discern the amino acid sequence and the exact site(s) of phosphorylation. By CID analysis we identified and confirmed the amino acid sequence of the peptide RAS^{982}VKNVDEKSGADGKPGTM and revealed the presence of a phosphate group at Ser^{982}. Moreover, we also found the spectra of the unmodified peptide as well as both the doubly and triply charged phosphopeptides (Figures 9C and D). Importantly, only the unphosphorylated peptide RAS^{982}VKNVDEKSGADGKPGTM was detected in TRP isolated from $inaC^{p209}$ flies. Based on these data, we conclude that TRP is phosphorylated *in vivo* at Ser^{982} by eye-PKC**.**

trp^{S982A} displays slow deactivation of the visual response

To gain insight into the functional significance of TRP phosphorylation at Ser^{982}, we generated and characterized transgenic flies expressing a modified *trp*, trp^{S982A}, in which the phosphorylation site is eliminated. As a control, we also generated transgenic flies expressing a wild-type *trp* (trp^{wt}). The expression of wild-type or modified *trp* was under the control of the *hsp70* promoter, and the function of TRP was analyzed in a null

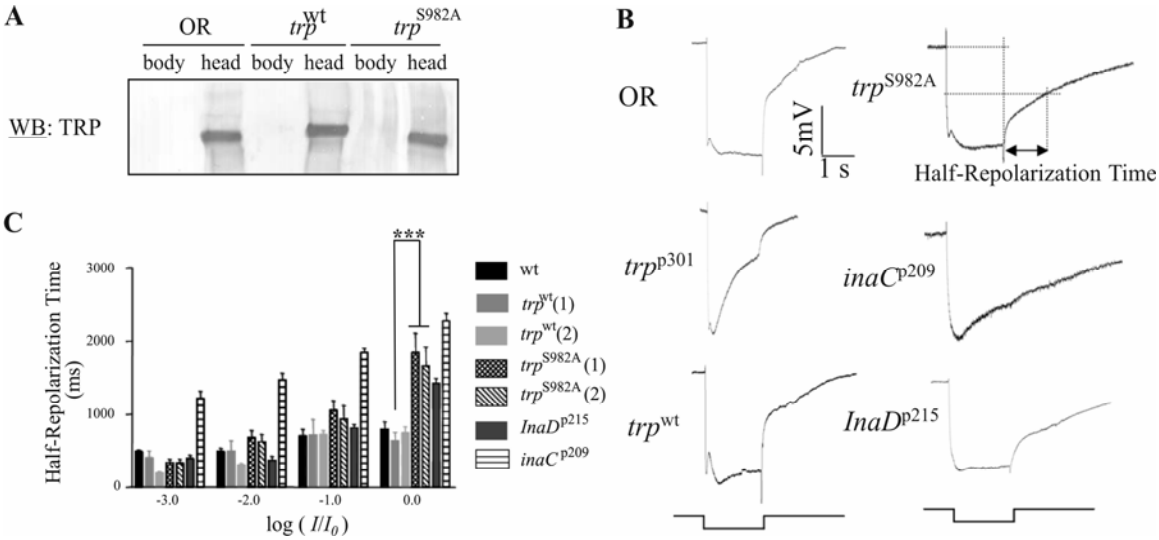


Figure 10. Biochemical and electrophysiological characterization of transgenic flies lacking the phosphorylation site at Ser^{982}. (**A**) Western blotting. The expression of TRP in the fly head or body was analyzed. (**B**) ERG analysis. Shown are representative ERG recordings of wild-type (OR), *trp*^{p301}, *trp*^{wt}, *trp*^{S982A}, *inaC*^{p209} and *InaD*^{p215} flies following stimulation of a two-second pulse of the brightest light stimulus (log I/I_0 = 0). (**C**) A histogram that compares half-repolarization times (n = 5, mean $\pm$ S.E.M.) at different light stimuli. The stimuli were 2 sec white lights without any attenuation (log I/I_0 = 0, I = stimulus intensity used, I_0 = maximum stimulus intensity available) or attenuated by one (log I/I_0 = -1), two (log I/I_0 = -2) or three (log I/I_0 = -3) log units. The half-repolarization time is the time required to reach 50% of repolarization, as diagrammatically depicted for *trp*^{S982A} flies in (**B**). Two independent transgenic lines for both *trp*^{wt} and *trp*^{S982A} were used for quantification**.** *** p = 0.001.

genetic background (trp^{p301} (*35, 42*)). We first determined if the modified TRP is stably expressed by Western blotting. Indeed, we observed that the TRP protein in trp^{S982A} flies reaches a steady-state concentration similar to that of wild-type flies (Oregon-R, OR) or transgenic flies expressing a wild-type *trp*, trp^{wt} (Figure 10A). It appears that basal transcription driven by the *hsp70* promoter is sufficient for transcription of *trp* leading to wild-type level of TRP in trp^{wt} and trp^{S982A} flies.

Next we characterized the visual electrophysiology by ERG for gaining insight into the *in vivo* activity of the modified TRP. ERG is an extracellular recording of the compound eye. Briefly, red-eye flies were dark-adapted for two minutes and then given a two-second white light stimulation. Using this stimulation paradigm, wild-type flies displayed the characteristic ERG waveform consisting of fast depolarization, maintained depolarization, and fast repolarization components (Figure 10B). In contrast, trp^{p301} flies displayed the initial fast depolarization but lacked the maintained component, and therefore the membrane potential returned gradually to baseline. This abnormal phenotype of trp^{p301} was completely rescued by transgenic expression of wild-type *trp* (Figure 10B). Remarkably, transgenic expression of trp^{S982A} rescued the trp^{p301} phenotype but with delayed deactivation kinetics (Figure 10B). Close inspection of the deactivation kinetics in ERG revealed two subcomponents: a fast and a slow component. The fast subcomponent occurs immediately following light termination and achieves over 50% repolarization. The fast subcomponent is followed by the slow subcomponent that eventually returns the potential to baseline. It appears that trp^{S982A} flies exhibit defects in the fast subcomponent.

To further characterize *trp*^{S982A} flies we examined their visual response to various intensities of light over 4 log units. We show that the prolonged deactivation kinetics is more prominent during the brightest light stimulation (log I/I_0 = 0): the half-repolarization time of *trp*^{S982A} is approximately two-fold longer than that of wild-type flies (Figure 10C, wild-type, 0.801 ± 0.119 s; *trp*^{wt}, 0.842 ± 0.064 s; *trp*^{S982A}, 1.668 ± 0.253 s, n = 5, mean ± S.E.M.). In contrast, the amplitude of the ERG responses in *trp*^{S982A} was comparable to that of *trp*^{wt} flies (*trp*^{wt}, 18.008 ± 0.95 mV; *trp*^{S982A}, 20.71 ± 2.73 mV, n = 5, mean ± S.E.M.), indicating that activation of visual signaling is not affected in *trp*^{S982A}. These results demonstrate that expression of *trp*^{S982A} leads to slow deactivation of visual response, which is likely due to a loss of eye-PKC phosphorylation of the modified TRP.

We compared the deactivation kinetics of *trp*^{S982A} with that of *inaC*^{p209} flies that lack eye-PKC. Interestingly, *inaC*^{p209} exhibited prolonged deactivation kinetics similar to *trp*^{S982A}, in response to bright light stimuli. However, *inaC*^{p209} also shows defects in deactivation at lower light intensities: the half-repolarization time was at least two fold longer than that of wild-type, regardless of the light intensity used (log I/I_0 = 0, *inaC*^{p209}, 2.216 ± 0.1 s; log I/I_0 = -1, wild-type, 0.708 ± 0.087 s, *trp*^{wt}, 0.721 ± 0.209 s, *trp*^{S982A}, 0.938 ± 0.182 s, *inaC*^{p209}, 1.852 ± 0.053 s; log I/I_0 = -2, wild-type, 0.491 ± 0.044 s, *trp*^{wt}, 0.501 ± 0.139 s, *trp*^{S982A}, 0.626 ± 0.102 s, *inaC*^{p209}, 1.493 ± 0.091 s; log I/I_0 = -3, wild-type, 0.490 ± 0.087 s, *trp*^{wt}, 0.410 ± 0.091 s, *trp*^{S982A}, 0.337 ± 0.048 s, *inaC*^{p209}, 1.219 ± 0.1 s, n = 5, mean ± S.E.M.). These results indicate that the deactivation defect in *inaC*^{p209} is more complex than that of *trp*^{S982A} and suggest that phosphorylation of

additional PKC sites in TRP or other substrates may be responsible for the fast deactivation of the visual response.

We also investigated the deactivation kinetics of trp^{S982A} in comparison with that of $InaD^{p215}$ (*139*). $InaD^{p215}$ contains a modified INAD, $INAD^{M442K}$, which fails to associate with TRP (*42*). The lack of the TRP-INAD interaction leads to a slow recovery of the visual response (*42*). We found that $InaD^{p215}$ displayed an ERG phenotype similar to that of trp^{S982A} with deactivation defects that manifested at bright light stimulation. Moreover, as for trp^{S982A}, the deactivation kinetics of $InaD^{p215}$ at low light intensities were indistinguishable from wild-type (Figure 10C, half-repolarization time for $InaD^{p215}$, log I/I_0 = 0, 1.432 ± 0.064 s; log I/I_0 = -1, 0.815 ± 0.045 s, log I/I_0 = -2, 0.374 ± 0.052 s, log I/I_0 = -3, 0.398 ± 0.041 s, n = 5, mean ± S.E.M.). These results indicate that the slow recovery of $InaD^{p215}$ may be due to a loss of eye-PKC phosphorylation in TRP.

Taken together, our biochemical and electrophysiological analyses demonstrate that phosphorylation of TRP at Ser^{982} by eye-PKC is important for the rapid deactivation of visual signaling in *Drosophila.*

Discussion

Reversible phosphorylation modulates the dynamics of signal transduction by transiently altering activities of signaling proteins. Members of the conventional PKC family (*102*), which are activated by Ca^{2+} and DAG, are capable of phosphorylating a wide variety of protein substrates for temporal and spatial regulation of signaling processes (*141*). In *Drosophila*, eye-PKC is involved in the negative regulation of visual signaling as $inaC^{p209}$ flies lacking eye-PKC display abnormal desensitization, slow

deactivation and defects in light adaptation (*31, 142*). Eye-PKC is anchored to a macromolecular complex by tethering to INAD (*46, 54*). Interaction with INAD enhances the stability of eye-PKC as well as targets eye-PKC to the rhabdomeres of photoreceptors (*47*), where visual signaling occurs. Importantly, the *in vivo* function of eye-PKC is regulated by interaction with INAD. Previously, it was shown that eye-PKC phosphorylates TRP *in vitro* (*55, 57*). In the present study we investigated the molecular basis of TRP phosphorylation by eye-PKC.

To mimic eye-PKC phosphorylation of TRP *in vitro*, we designed a complex-dependent kinase assay. We demonstrated that the *in vitro* complex-specific phosphorylation of TRP is regulated by the presence of the INAD-interacting domain in TRP, as well as the existence of INAD in the fly extracts. We showed that extracts lacking either eye-PKC or INAD fail to support TRP phosphorylation. Similarly, extracts prepared from $InaD^{p215}$ that expresses a modified INAD devoid of the TRP binding (*42*), are not able to promote TRP phosphorylation. Together, these findings indicate that INAD targets eye-PKC to its substrates, similar to a receptor for activated C kinase (RACK) (*55*). By the complex-dependent kinase assay, we identified Ser^{982} of TRP as an eye-PKC phosphorylation site. Moreover, we analyzed TRP isolated from flies by LC-MS and found that Ser^{982} of TRP is indeed phosphorylated *in vivo* by eye-PKC because phosphorylated peptides encompassing Ser^{982} of TRP were present in wild-type, but absent in $inaC^{p209}$ flies.

Next, we investigated the *in vivo* functional contribution of phosphorylation by characterizing transgenic flies expressing a modified TRP bearing an Ala substitution at Ser^{982} (trp^{S982A}). Remarkably, these transgenic flies displayed prolonged deactivation

kinetics in response to bright light stimuli, indicating that phosphorylation of TRP at Ser^{982} by eye-PKC is involved in inactivation of TRP, leading to fast deactivation. A model of the TRP regulation by eye-PKC is proposed (Figure 11). TRP is an integral part of the INAD complex and is opened by light. Following light termination, the visual response is rapidly deactivated. Although molecular mechanisms underlying deactivation remain elusive, Ca^{2+} is known to play a vital role in response termination (*32, 61-63*). The increased intracellular Ca^{2+} (primarily mediated by TRP) and DAG activate eye-PKC, which, in turn, phosphorylates TRP at Ser^{982}. Phosphorylation of TRP leads to a rapid inactivation of the channel upon cessation of the light stimulation (Figure 11B), without affecting the interaction between TRP and INAD (data not shown). How does phosphorylation influence the TRP channel activity? Ser^{982} is located within the Lys-Pro rich region of TRP (Figure 9B), which may function in TRP gating (*41*). We speculate that phosphorylation at Ser^{982} may induce a conformational change in the pore domain, which in turn leads to a rapid closure and inactivation of TRP. Phosphorylation has been linked directly to conformational changes that play key roles in the regulation of ion channels (*144*). It is also possible that phosphorylation of TRP at Ser^{982} affects the interaction with some yet unidentified proteins which may be important for the modulation of the TRP channel activity.

In the absence of eye-PKC-mediated phosphorylation of TRP, deactivation of visual signaling is slower as observed in $inaC^{p209}$ or trp^{S982A}. We found that $inaC^{p209}$ displays a more complex deactivation defect, whereas trp^{S982A} exhibits prolonged deactivation only in response to bright light. These findings suggest that in addition to

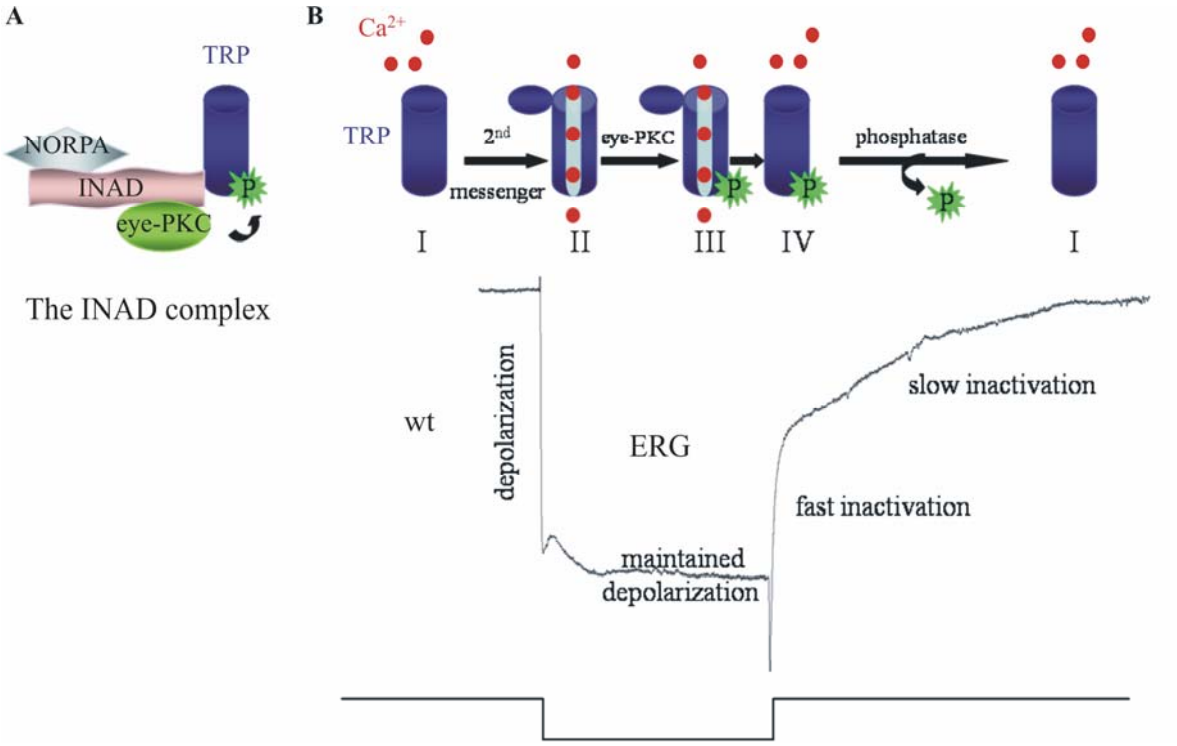


Figure 11. A model of the TRP regulation by eye-PKC-mediated phosphorylation at Ser^{982}. (**A**) INAD facilitates phosphorylation of TRP at Ser^{982} by eye-PKC, by positioning eye-PKC in close proximity to TRP. (**B**) Resting TRP channels (**I**) become open in response to light stimulation (**II**) leading to a massive influx of Ca^{2+} that results in depolarization of the photoreceptor cells. Meanwhile, increases in the concentrations of intracellular Ca^{2+} and DAG activate eye-PKC, which, in turn, phosphorylates TRP (**III**). When light stimulation is off, phosphorylated TRP rapidly becomes inactivated (**IV**)**.** It is likely that eye-PKC-mediated phosphorylation at Ser^{982} of TRP facilitates the conformational changes associated with the closure of the channel. To return the TRP channels to the resting state, dephosphorylation with the participation of protein phosphatases may occur. Shown at the bottom is a representative ERG of a wild-type fly.

TRP, eye-PKC phosphorylates other substrates for efficient termination of the light response. Indeed, eye-PKC has been shown to phosphorylate INAD (*56, 55*) but the functional relevance of this phosphorylation is not known. Furthermore, Gu *et al*. (2005) reported that eye-PKC is required for the Ca^{2+}-dependent inhibition of NORPA. NORPA is part of the INAD complex, however, it is not known to be phosphorylated by eye-PKC. Gu *et al.* (2005) also showed that the Ca^{2+}-dependent inactivation of the light-induced current was unaltered in $inaC^{p209}$. This finding suggests the existence of a parallel Ca^{2+}-dependent mechanism in $inaC^{p209}$ by which TRP is inactivated or of an upregulation of a Ca^{2+}-dependent mechanism that activates other kinases (*71*) to compensate for the loss of eye-PKC in $inaC^{p209}$.

Importantly, trp^{S982A} displays slow deactivation kinetics similar to that of $InaD^{p215}$. $InaD^{p215}$ was isolated by Pak *et al.* (*139*) based on the *ina* (inactivation-no-afterpotential) phenotype elicited by ERG. By whole-cell recordings, Shieh and Niemeyer (*137*) showed that $InaD^{p215}$ exhibits slow deactivation kinetics. However, Tsunoda *et al*. (*46*) reported a delay in latency of the quantum bump and proposed that activation was affected in the $InaD^{p215}$ mutant. To resolve this discrepancy, Henderson *et al.* (*138*) re-examined the mutant and concluded that the primary defect in $InaD^{p215}$ is prolonged deactivation and not slow activation. $InaD^{p215}$ expresses $INAD^{M442K}$ that fails to associate with TRP (*137*). How does a loss of INAD-TRP interaction lead to abnormal deactivation of visual signaling? It is likely that the lack of the INAD-TRP interaction prevents the recruitment of TRP to the INAD complex and consequently, eye-PKC-mediated regulation. Indeed, both trp^{S982A} and $InaD^{p215}$ exhibit similar deactivation defects, indicating that the molecular basis underlying the slow deactivation defect in

InaD^{p215} is due to a lack of negative regulation of the TRP channel by eye-PKC. Together, these findings suggest that formation of the INAD complex is essential for fast deactivation of the visual response by promoting phosphorylation of TRP by eye-PKC. Moreover, Ser^{982} may be the sole eye-PKC phosphorylation site in TRP because *trp*^{S982A} and *InaD*^{p215} display similar deactivation defects. A loss of INAD-TRP interaction was previously investigated in transgenic flies expressing modified TRP in which the INAD-interacting domain was deleted (*trp*$^{\Delta 1272}$). Li and Montell (2000) reported a reduced light response with normal deactivation kinetics in *trp*$^{\Delta 1272}$. These authors proposed that the suppression of the delayed termination, which is due to a reduced eye-PKC level in *trp*$^{\Delta 1272}$ is probably masked by a concomitant decrease in TRP and INAD levels (*40*).

To date many proteins related to *Drosophila* TRP have been discovered in both invertebrates and vertebrates. These TRP ion channels are subdivided into seven subfamilies (TRPC, TRPV, TRPM, TRPN, TRPA, TRPP, and TRPML) (*37*). *Drosophila* TRP belongs to the TRPC subfamily. Members of the TRPC subfamily are also activated by receptor-induced activation of phospholipase C (*14*) and therefore, may be regulated by PKC. Indeed, phosphorylation of the TRPC channels by PKC appears important for modulating the channel activity. For example, the PKC-mediated phosphorylation of TRPC1 was shown to contribute to its SOC activation, triggering Ca^{2+} entry into endothelial cells (*79*). In contrast, PKC-mediated phosphorylation was demonstrated to inhibit the activity of TRPC3 in HEK 293 cells (*83*) and of TRPC6 in PC12D neuronal cells (*86*). In both cases, TRPC3 and TRPC6 are activated by DAG, whereas DAG also turns on PKC. The authors proposed that timing is important because the channels are activated by DAG more rapidly than they are inhibited by DAG-activated PKC (*86*).

Heterologously expressed TRPC7 was also shown to be regulated by PKC: inhibition of PKC prolonged inactivation of the channel (*88*). Moreover, PKC phosphorylation of heterologously expressed TRPC5 resulted in desensitization of this channel, a process that was dependent on both extracellular and intracellular Ca^{2+} concentrations (*85*).

In conclusion, here we uncover the molecular mechanism underlying the complex-dependent phosphorylation of TRP by eye-PKC and its role in fast deactivation of vision. Specifically, we show that eye-PKC phosphorylates TRP at Ser^{982} *in vitro* and *in vivo*. Importantly, phosphorylation of TRP facilitates rapid inactivation of the channel because transgenic flies bearing an Ala substitution at Ser^{982} display prolonged deactivation kinetics of the light response. Significantly, this slow deactivation defect is similar to that observed in $InaD^{p215}$ in which TRP fails to associate with INAD. Our findings provide insights into the mechanistic basis of slow deactivation in $InaD^{p215}$, suggesting that INAD plays a critical role in targeting eye-PKC to TRP for rapid deactivation of the visual signaling. Taken together, these data indicate that the INAD macromolecular complex is important for deactivation of the visual response by directing eye-PKC to TRP. Furthermore, PKC-mediated phosphorylation of TRP at Ser^{982} leads to fast deactivation of vision by promoting inactivation of the TRP channel.

CHAPTER III

INVESTIGATION OF OTHER POTENTIAL EYE-PKC PHOSPHORYLATION SITES IN TRP

Introduction

Drosophila TRP is a light-activated Ca^{2+} channel that plays a crucial role in the visual signaling pathway. TRP is a member of the TRP channel superfamily, which is subdivided into six subfamilies (TRPC, TRPV, TRPM and TRPN, TRPA, TRPP, and TRPML) (*37*). *Drosophila* TRP belongs to the TRPC subfamily and contains an extended C-terminus. TRPC channels are activated upon stimulation of PLCβ. Some TRPC members are known to be SOCs, whereas others are activated by DAG (see Chapter I). However, the gating mechanism of *Drosophila* TRP has not been firmly established. Potential secondary messengers include DAG (*28*) and PUFAs *(29)*.

TRP is responsible for 90% of photoreceptor depolarization (*35, 43, 146-148*). Once the light stimulus ceases, inactivation of both TRP and TRPL channels, and hence deactivation, occurs in less than 20 ms (*52*). The high temporal resolution of visual signaling may be due to the existence of INAD complexes, which promote the correct subcellular localization of proteins involved in visual signaling. Eye-PKC plays an important role in deactivation of visual signaling, since flies lacking eye-PKC ($inaC^{P209}$) display slow deactivation kinetics and also undergo light-dependent retinal degeneration (*31*). Eye-PKC shares 52% amino acid identity with human PKCα and is thus a member of the conventional PKC family that is activated by both DAG and Ca^{2+}. The *in vivo* function of eye-PKC appears to be dependent upon its interaction with INAD, since a loss of interaction results in an ERG phenotype similar to that of $inaC^{P209}$ flies (*54*). This

result strongly suggests that eye-PKC regulates proteins in the INAD complex. Indeed, eye-PKC was shown to phosphorylate the TRP channel *in vitro* in *Drosophila* (*55*) and *Calliphora* (*75*). By complex-dependent kinase assays and mass spectrometry analysis, we have demonstrated previously that eye-PKC phosphorylates TRP at Ser^{982} both *in vitro* and *in vivo*, and that phosphorylation at this site is important for deactivation of visual signaling, because mutant flies lacking this phosphorylation site exhibit prolonged deactivation kinetics by ERG recordings (see Chapter II). Specifically, trp^{S982A} flies displayed defects in the rapid component of deactivation in response to bright light, whereas their response to low intensities of light was normal (see Chapter II). In contrast to trp^{S884A}, $inaC^{p209}$ exhibited a more profound deactivation defect that manifested itself regardless of the light intensity stimulus used (see Chapter II). These data suggest that there is another eye-PKC-dependent mechanism that mediates deactivation of visual signaling. This mechanism may involve phosphorylation of another amino acid in TRP. In our previous *in vitro* phosphorylation studies we were not able to evaluate the intracellular C-terminal region of TRP upstream of amino acid 906, because we failed to produce stable fusion proteins in bacteria (see Chapter II). Interestingly, within this region there are eight putative PKC phosphorylation sites as predicted by NetPhos and Prosite software, using the consensus PKC motif (S/T)-X-(R/K). Moreover, only three of the eight phosphorylation sites were covered, and shown not to be phosphorylated *in vivo*, in our previous mass spectrometry analysis.

Here we investigated whether there is an additional PKC phosphorylation site in the intracellular C-terminal sequence of TRP upstream of amino acid 906. We employed *in vitro* kinase assays using GST-TRP fusion peptides encompassing the putative PKC

sites as substrates, and recombinant human PKCα as the enzyme. We found that a GST-peptide containing two putative PKC sites, Ser^{881} and Ser^{884}, was highly phosphorylated by PKCα. Using site-directed mutagenesis we determined that Ser^{884} was highly phosphorylated *in vitro* by PKCα. An alignment of TRP sequences from various invertebrate species revealed that this residue is highly conserved. In addition, Ser^{884} is positioned close to the calmodulin-binding domain of TRP. Taken together, these data suggested that phosphorylation of Ser^{884} may be functionally important. We assessed the functional relevance of this phosphorylation site by generating transgenic flies expressing a modified TRP in which Ser^{884} was replaced with Ala. Transgenic flies were analyzed by ERG. Preliminary data show that trp^{S884A} exhibits normal kinetics in response to light, indicating that phosphorylation of Ser^{884} in TRP by PKC does not contribute to regulation of the light response in *Drosophila*.

Materials and Methods

Generation of GST-TRP fusion peptides

Synthetic oligonucleotides encoding each of the predicted PKC sites were designed and generated, as follows:

Ser^{669} 5’ AATTCTACCAAATCATCTCGGAGCGAGCCGACTAAC 3’ (sense)

5’ TCGAGTTAGTCGGCTCGCTCCGAGATGATTTGGTAG 3’ (antisense)

Ser^{772} 5’ AATTCCGCCAGGACATCAGCTCCTTGCGGTTCGAGTAAC 3’ (sense)

5’ TCGAGTTACTCGAACCGCAAGGAGCTGATGTCCTGGCGG 3’ (antisense)

Thr^{801} 5’ AATTCGGAGTTGCTCGAACCACCAAGGGCAAGTAAC 3’ (sense)

5’ TCGAGTTACTTGCCCTTGGTGGTTCGAGCAACTCCG 3’ (antisense)

Ser^{832} 5’ AATTCAGCGAATCTGAGAGCGGACGAGATATATAAC 3’ (sense)

5’ TCGAGTTATATATCTCGTCCGCTCTCAGATTCGCTG 3’ (antisense)

Thr^{849} 5’ AATTCGGCAGAAAGAAGACCCAGAAGGGAGACTAAC 3’ (sense)

5’ TCGAGTTAGTCTCCCTTCTGGGTCTTCTTTCTGCCG 3’ (antisense)

Ser^{872} 5’ AATTCGATCCCATTGGCTCCAAGCGCTCCTCCTAAC 3’ (sense)

5’ TCGAGTTAGGAGGAGCGCTTGGAGCCAATGGGATCG 3’ (antisense)

Ser^{881} 5’ AATTCATGCAACGTCATAGCCAGCGAAGCTTGTAAC 3’(sense)

5’ TCGAGTTACAAGCTTCGCTGGCTATGACGTTGCATG 3’(antisense)

Ser^{884} 5’ AATTCCGTCATAGCCAGCGAAGCTTGAGGAGGAAGTAAC 3’ (sense)

3’ TCGAGTTACTTCCTCCTCAAGCTTCGCTGGCTATGACGG 3’ (antisense)

Ser^{881} and Ser^{884} were converted to Ala residues using the following synthetic oligonucleotides:

S881A 5’ AATTCCGTCATGCCCAGCGAAGCTTGAGGAGGAAGTAAC 3’ (sense)

5’ TCGAGTTACTTCCTCCTCAAGCTTCGCTGGGCATGACGG 3’ (antisense)

S884A 5’ AATTCCGTCATAGCCAGCGAGCCTTGAGGAGGAAGTAAC 3’ (sense)

5’ TCGAGTTACTTCCTCCTCAAGGCTCGCTGGCTATGACGG 3’(antisense)

S881A, S884A

5’ AATTCCGTCATGCCCAGCGAGCCTTGAGGAGGAAGTAAC 3’ (sense)

3’ TCGAGTTACTTCCTCCTCAAGGCTCGCTGGGCATGACGG 3’ (antisense)

35 ng of both the sense and complementary antisense oligonucleotides were first annealed in a buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM DTT, 0.1 mM EDTA, and 50% glycerol, by incubating at 60^0 C for two minutes, and gradually cooling the

tubes to room temperature. The annealed oligonucleotides were ligated into EcoRI and XhoI sites of pGEX-4T1. Next, *Escherichia coli* HMS174 cells were transformed with pGEX-4T1 constructs. Overnight cultures (1.5 ml) from a single colony were used to inoculate 50 ml of Luria-Broth containing ampicillin (50μg/ml). Cultures were grown at 37^{o}C for 2-3 hours until the density of bacterial cultures (OD_{600}) reached 0.6-0.8. Expression of fusion peptides was initiated by adding IPTG to a final concentration of 0.1-1 mM). Cultures were harvested 3 hours after induction, and bacterial pellets were collected by centrifugation. Bacterial lysates containing the fusion peptides were prepared by re-suspending pellets in Extraction Buffer (EB) (1X Phosphate Buffered Saline (PBS), 10 mM DTT, 10 mM EDTA, 1% Triton X-100, and a mixture of protease inhibitors containing 1 mM PMSF, 1 μg/μl leupeptin and pepstatin A, 5 μg/μl aprotinin, 0.01 mM benzamide, 1 mM benzamidine). Re-suspended pellets were subjected to 20-30 strokes on ice using a Polytron homogenizer, and incubated at 4°C with constant agitation for 1 h to extract GST-fusion peptides. The mixture was centrifuged at 12,000 rpm for 20 min at 4°C, and the supernatant containing GST-fusion peptides was used for immobilizing the GST-fusion peptides on Glutathione beads.

In vitro kinase assay

In vitro kinase assays were performed as described in Chapter II.

LC-MS analysis

LC-MS was performed by the Vanderbilt Mass Spectrometry Research Center, as described previously (see Chapter II, Materials and Methods).

Fly stocks

Fly stocks (*Drosophila melanogaster*) were maintained at 25°C in a 12/12-hr light/dark cycle. The wild-type strain was Oregon-R and the mutant included trp^{p301}.

P-element-mediated Germ-line Transformation

Modified *trp* cDNA was subcloned into a pCaSpeR 4 vector that contains the *Drosophila hsp70* promoter. The P-element construct and a transposase plasmid ("wings-clipped") were injected into *y [1] w[67c23]* embryos (CBRC transgenic *Drosophila* Core, Massachusetts General Hospital- Harvard Medical School). Crosses were carried out using standard techniques. Flies with the transgene integrated into the X and second chromosomes were selected and made homozygous in the trp^{p301} background for further analysis.

WB analysis

WB was performed using alkaline phosphatase-conjugated secondary antibodies. Antigens were visualized upon staining with BCIP and NBT. Polyclonal antibodies against INAD and TRP were generated as described before (*42, 137*).

ERG Recordings

ERG recordings were performed as described in Chapter II.

Results

Ser^{884} of TRP is phosphorylated *in vitro* by PKCα

To gain better insight into the regulation of TRP by eye-PKC, we focused our attention on the previously unexamined intracellular C-terminal sequence of TRP. Using NetPhos and Prosite software, and the PKC consensus sequence motif (S/T)-X-(R/K), we identified eight putative PKC phosphorylation sites within the TRP sequence between amino acid residues 657 and 906. To determine whether any of these potential PKC sites are indeed PKC phosphorylation sites, we generated GST-fusion peptides spanning each of these putative PKC sites, and assessed phosphorylation by recombinant human PKCα. We found that the GST-TRP$^{779\text{-}888}$ fusion peptide was phosphorylated by PKCα, whereas the negative control, GST, was not. The phosphorylation of other GST-TRP peptides was negligible (Figure 12).

The sequence spanning TRP$^{779\text{-}888}$ contains two putative PKC sites, Ser^{881} and Ser^{884}. To investigate which of the two sites are targeted by PKCα, we used site-directed mutagenesis to substitute one or both of the PKC sites with Ala. We show that phosphorylation of the TRP$^{779\text{-}888}$ peptide lacking Ser^{884} was reduced by 80%, whereas the phosphorylation of the peptide lacking Ser^{881} was decreased by only 26% (Figure 13A). These data demonstrate that Ser^{884} of TRP can be phosphorylated *in vitro* by PKCα.

Is Ser^{884} of TRP phosphorylated by eye-PKC?

Once we established that Ser^{884} is phosphorylated by PKCα, we explored whether eye-PKC could also phosphorylate Ser^{884}. However, we were not able to test whether Ser^{884} is phosphorylated by eye-PKC *in vitro* because TRP$^{779-888}$ lacks the PDZ-binding region required for the TRP-INAD interaction. In addition, as mentioned previously, the fusion proteins containing sequences spanning TRP$^{657-1275}$ is not stable in *E. coli.* Thus, in both cases, the complex-dependent kinase assay could not be employed to evaluate the eye-PKC-dependent phosphorylation at Ser^{884} in TRP.

We investigated whether Ser^{884} is phosphorylated *in vivo* by eye-PKC, by LC-MS. The TRP channel was isolated from light-adapted wild-type flies by immunoprecipitation using anti-INAD antibodies. Proteins in the complex were resolved by SDS-PAGE, and stained with Colloidal Blue. The band corresponding to TRP was excised, digested in-gel using trypsin or chymotrypsin. This mixture of peptides was subjected to LC-MS, and resultant data were analyzed by the SEQUEST algorithm, as described in Chapter II. Unfortunately, the TRP sequence between amino acid residues 879 and 887 was not identified by SEQUEST. One possible reason for this is that complete digestion of the sequence surrounding Ser^{884} (MQRHSQRS^{884} LRRKII), using either trypsin (cleaves at C-terminal of K or R) or chymotrypsin (theoretically, cleaves at C-terminal of F, W, Y , but experimentally, also cleaves nonspecifically at K, R, L, , N, Q, C, H, S, and M), may yield peptides containing less than five amino acid residues that are beyond the limits of MS detection. To bypass this potential problem, we performed a partial digestion of TRP, for 15, 30 and 60 minutes; however, the region encompassing Ser^{884} was still not covered by SEQUEST, and therefore, its *in vivo* phosphorylation state

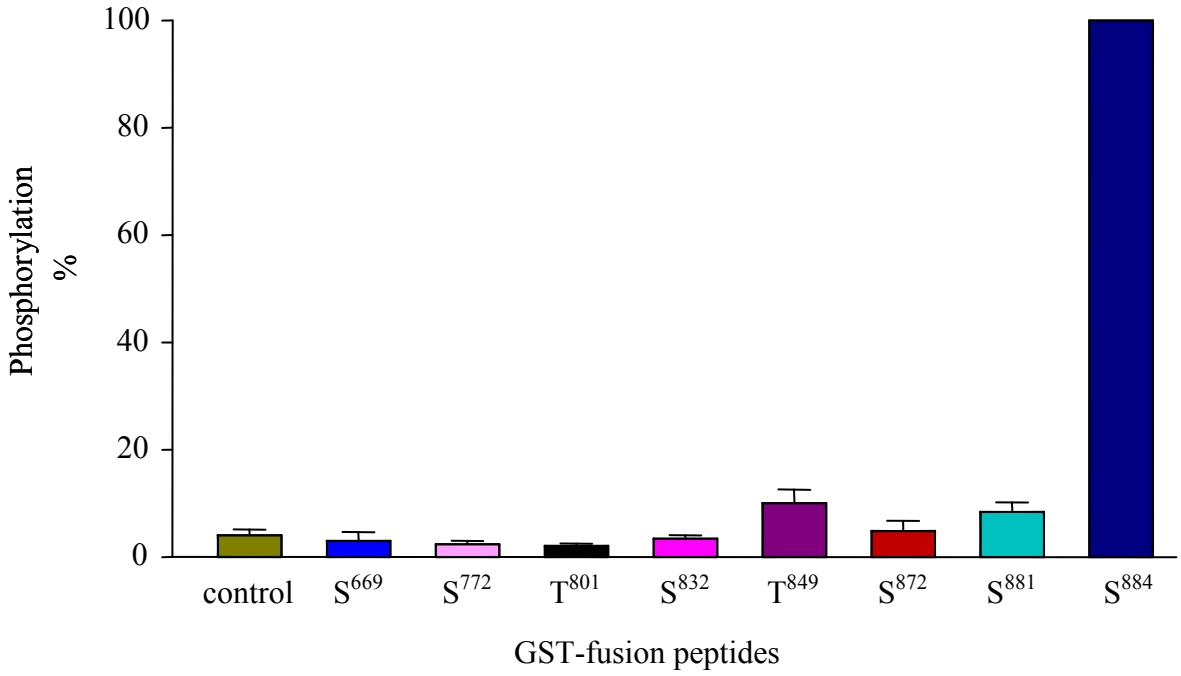
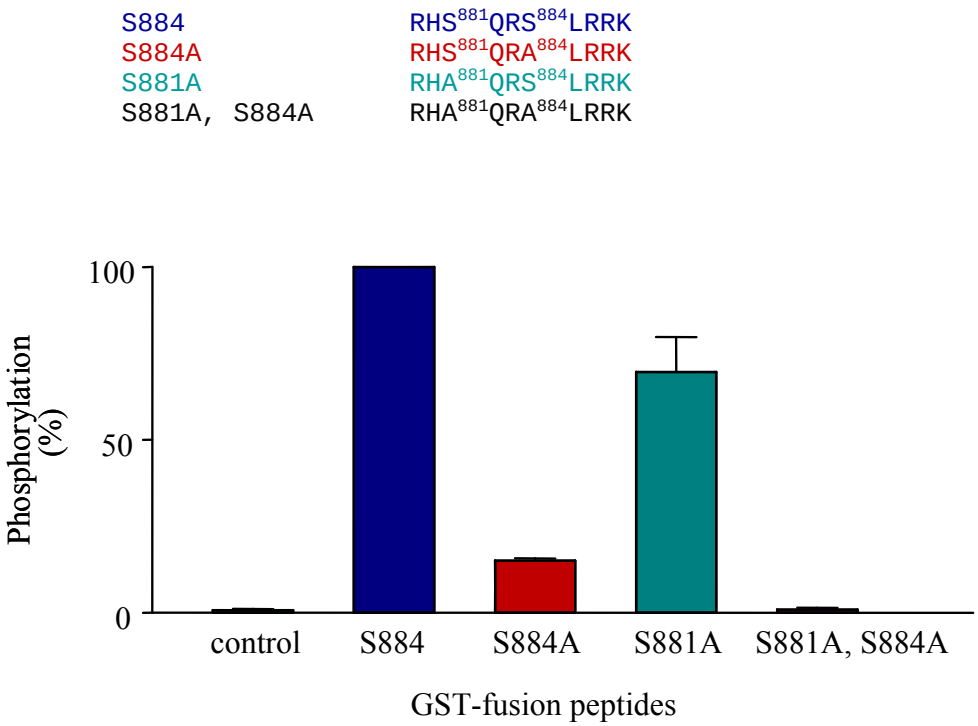


Figure 12. The peptide containing Ser^{884} is phosphorylated *in vitro* by recombinant PKCα. The TRP peptides were designed as described in *Materials and Methods*. These peptides contain putative PKC sites within the TRP C-terminal region upstream of amino acid 906. Putative PKC sites were identified using NetPhos and Prosite software, and the PKC consensus motif (S/T)-X-(L/K). control, GST, $n = 3$.

A



B

dmTRP	878	Q R H S Q R S L R R K I I E
cvTRP	886	Q R H S Q R S L R R R I I D
agTRP	828	M R R S R Q S L R K Q I L D
amTRP	867	S I Q N Q I S L K K A M E A

Figure 13. Ser^{884} in TRP is phosphorylated *in vitro* by PKCα. (**A**) Peptide sequences used in the *in vitro* kinase assay are shown above. The histogram, which indicates that Ser^{884} is phosphorylated *in vitro* by PKCα, is shown below. The incorporated radioactivity was normalized to protein levels obtained by densitometry (Odyssey analysis software), and the relative phosphorylation was presented as mean ± S.D. (n = 3). (**B**) Ser^{884} (*blue*) of *Drosophila* TRP is conserved across insect species. Residues corresponding to Ser^{881} and Ser^{884} are denoted by the black rectangles. dm, *Drosophila melanogaster*, cv, *Calliphora vicinia*, ag, *Anophelus gambiae*, am, *Apis meliphera*.

remains elusive. Although we were unable to demonstrate the phosphorylation of Ser^{884} by eye-PKC, we noticed that Ser^{884} is conserved across different insect species (Figure 13B), suggesting a functional role for this residue.

trp^{S884A} expressing the wild-type level of TRP^{S884A} displays normal kinetics by ERG recordings

To address the functional significance of phosphorylation of Ser^{884} in TRP by PKC, we generated transgenic flies expressing a modified TRP lacking this phosphorylation site. These flies, *trp*^{S884A}, express a modified TRP in which Ser^{884} is substituted with Ala, which is under the control of the *hsp70* promoter. The function of TRP^{S884A} was explored in a *trp* null genetic background (*trp*^{p301}). By Western blotting, we examined the expression of TRP^{S884A} and observed that the basal expression of TRP^{S884A} in young (1 day) and old (10 day) *trp*^{S884A} flies was variable: some transgenic flies displayed wild-type levels of TRP, whereas others exhibited no detectable TRP (Figure 14A).

We characterized the functional consequence of the loss of PKC phosphorylation at Ser^{884} in TRP using ERG, an extracellular recording method in which the electrical signals from the whole eye are recorded. A normal ERG waveform consists of initial fast depolarization, maintained depolarization and fast repolarization components. Unexpectedly, the ERG phenotype of *trp*^{S884A} was variable, ranging from wild-type to abnormal waveforms. Significantly, the visual phenotype correlated with the levels of TRP in transgenic flies: flies containing wild-type amounts of TRP displayed a wild-type ERG waveform, whereas flies without detectable levels of TRP exhibited an abnormal ERG waveform similar to that of mutant flies lacking TRP, *trp*^{p301} (Figure 14B).

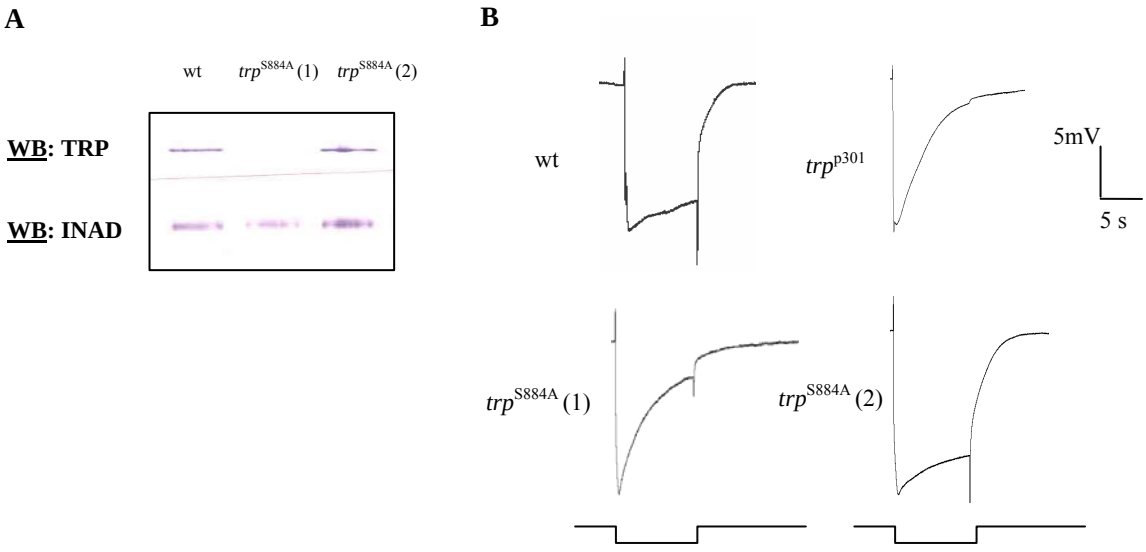


Figure 14. *trp*^{S884A} expressing wild-type level of TRP displays normal response to light stimulation. (A) Western blotting showing variable expression of TRP in young (1 day) *trp*^{S884A} flies (*trp*^{S884A}(1) and *trp*^{S884A}(2)). (**B**) ERG analysis. Shown are representative ERG recordings of wild-type (wt), *trp*^{p301}, and two *trp*^{S884A} flies whose Western blotting is shown in (**A**). A white light pulse of 10 s was used for stimulation.

Taken together, our preliminary biochemical and electrophysiological findings suggest that Ser^{884} of TRP does not play a role in regulation of the light response by eye-PKC, since flies expressing TRP^{S884A} exhibit a wild-type ERG phenotype. Further investigation is needed to provide an explanation for the variety of the biochemical and electrophysiological profiles of trp^{S884} flies.

Discussion

Deactivation of *Drosophila* visual signaling is a very fast process that likely involves multiple mechanisms. These mechanisms are Ca^{2+}–dependent, since by increasing the extracellular Ca^{2+}, the rate of deactivation is enhanced in isolated photoreceptor cells (*32, 61*). Remarkably, studies using fluorescent Ca^{2+} dyes show that the increase in intracellular Ca^{2+} is mediated primarily through light-activated TRP channels (*62*). The prime candidate for Ca^{2+}-mediated negative feedback of the light response is eye-PKC, a conventional PKC isoform that is activated by Ca^{2+} and DAG, and expressed specifically in the *Drosophila* eye. Indeed, the role of eye-PKC in deactivation of the light response and light adaptation has been demonstrated previously: flies lacking eye-PKC ($inaC^{p209}$) exhibited prolonged deactivation kinetics and defects in light adaptation (*31, 142*).

Importantly, eye-PKC is tethered to the scaffolding protein INAD (*54*), which also binds the TRP channel (*42*). INAD interacts constitutively with the main players involved in visual signaling including NORPA, eye-PKC and the TRP Ca^{2+} channel (*46, 149*). The anchoring of eye-PKC to INAD is mandatory for proper functioning of *Drosophila* visual signaling, since transgenic flies expressing eye-PKC^{I700D}, in which the

interaction of the kinase with INAD is eliminated, exhibit a profound defect in deactivation, similar to that of mutant flies lacking eye-PKC (*inaC*^{p209} flies) (*54*). The interaction between TRP and INAD is also important for phototransduction: mutant flies expressing modified INAD, in which a missense mutation (M442K) abolishes the interaction between INAD and TRP, display defects in termination of the light response (*137, 138*).

TRP was shown previously to be phosphorylated by eye-PKC *in vitro* (*75, 55*). In Chapter II we demonstrated that phosphorylation of TRP by eye-PKC *in vitro* depends on the anchoring of both proteins to INAD. In addition, we showed that eye-PKC phosphorylates TRP at Ser^{982}, and phosphorylation at this site is important for deactivation of the light response. Specifically, *trp*^{S982A} flies exhibited defects in deactivation kinetics in response to bright light, whereas their response to low light intensities was normal (see Chapter II). In contrast, flies lacking eye-PKC (*inaC*^{p209}) displayed more profound deactivation defects that manifested regardless of the light intensity stimulus used. These data indicate that eye-PKC mediates another event that contributes to deactivation of visual signaling, possibly by phosphorylating another amino acid in TRP.

In this study, we investigated whether there are any additional PKC sites in the previously unevaluated C-terminal sequence upstream of amino acid 906 of TRP. We generated GST fusion peptides spanning each putative PKC site, and demonstrated that Ser^{884} is heavily phosphorylated by recombinant PKCα. However, we were not able to test whether Ser^{884} is phosphorylated *in vitro* or *in vivo* by eye-PKC. We were also not able to assess the status of Ser^{884} phosphorylation *in vivo* by mass spectrometry, because

the spectra of peptides containing Ser^{884} were not recovered. Interestingly, Ser^{884} is conserved among several insect TRPs (Figure 13B), suggesting a functional role for Ser^{884}. Importantly, Ser^{884} is located close to the region that has been suggested to bind calmodulin (*48*).

We generated transgenic flies expressing modified TRP in which Ser^{884} was replaced by Ala. Our preliminary data indicate that phosphorylation of Ser^{884} does not have an effect on regulation of the light response, since trp^{S884A} flies display a normal ERG waveform in response to light. We also observed that some flies exhibited an abnormal ERG waveform lacking the maintained component (Figure 14B), however, this phenotype was apparently due to decreased levels of TRP^{S884A}. The variable TRP levels in trp^{S884A} flies may be due either to differential expression of TRP^{S884A}, or to age- or light-dependent protein degradation. Further investigation is needed to explore these possibilities.

Preliminary results of this study did not support that Ser^{884} is a candidate for the eye-PKC-dependent regulation of TRP. Therefore, the chapter regarding eye-PKC-mediated deactivation of *Drosophila* visual signaling remains open to investigation. Eye-PKC has been shown previously to phosphorylate other proteins that are involved in visual transduction, such as INAD (*56, 55*), but the functional relevance of this phosphorylation has not been investigated. Recently, NORPA has been suggested to be a substrate of eye-PKC based on electrophysiological studies (*150*), however, direct evidence of NORPA phosphorylation by eye-PKC is lacking.

Deactivation of visual signaling is a Ca^{2+}-dependent process. Other mechanisms that may be responsible for this Ca^{2+}-dependent process include 1) direct action of

calmodulin on the TRP channel, and 2) phosphorylation of TRP by Ca^{2+}/ calmodulin-dependent kinase II (CaMKII). Calmodulin is a small Ca^{2+} binding protein (148 amino acids) that acts as a primary sensor of intracellular Ca^{2+} changes. It can bind a maximum of four ions, and can exist as partially filled calmodulin or Ca^{2+}-free apocalmodulin. Ca^{2+}-bound calmodulin may have an effect on the activity of TRP, since TRP contains a putative calmodulin binding site, and has been shown to bind calmodulin in an overlay assay in the presence of Ca^{2+} (*48*). Moreover, CaMKII has been shown previously to phosphorylate Arr1 in *Drosophila* photoreceptors (*71, 72*). It is possible that CaMKII phosphorylates and regulates the TRP channel activity.

Summary and Future directions

Drosophila visual signaling is one of the fastest GPCR signaling pathways. Previously, we showed that eye-PKC phosphorylates the TRP Ca^{2+} channel at Ser^{982}, and this phosphorylation is important for deactivation of the light response. However, flies lacking eye-PKC ($inaC^{p209}$) exhibit a more severe defect in deactivation compared to trp^{S884A} flies, suggesting that eye-PKC phosphorylates another site in TRP besides Ser^{982}, or in another protein that plays a role in deactivation. In the current study we sought to determine if eye-PKC phosphorylates a second site in TRP, in addition to Ser^{982}. Our *in vitro* studies indicated that Ser^{884}, a conserved PKC site in TRP may be involved in the eye-PKC-dependent regulation of the visual response. However, studies using transgenic flies expressing TRP^{S884A} failed to support the possibility that phosphorylation at Ser^{884} of TRP plays a role in deactivation of the visual response.

CHAPTER IV

EYE-PKC IS PHOSPHORYLATED *IN VIVO* AT THR^{671}

Introduction

Drosophila eye-PKC is a photoreceptor-specific PKC isoform critical for light adaptation (*142*) and deactivation of the visual signaling (*31*). Eye-PKC binds to the second PDZ domain of INAD *via* its terminal PDZ-binding motif (*54*). A lack of interaction with INAD renders eye-PKC unable to regulate the visual signaling: transgenic flies expressing a modified eye-PKC (eye-PKC^{I700D}) in which the interaction of the kinase with INAD is eliminated, exhibit a defect in deactivation, similar to that of flies lacking eye-PKC (*inaC*^{p209}) (*54*).

PKC belongs to a family of serine/threonine-specific protein kinases that are implicated in diverse physiological and pathological processes (*98, 151*). As mentioned before, to date 12 different PKC isozymes have been identified in vertebrates. They are classified into three groups, according to the structure of the regulatory domain that consequently, determines the sensitivity to different cofactors. Conventional PKCs (α, βI, βII and γ) are activated by Ca^{2+} and DAG, whereas novel PKCs (δ, ε, η/L and θ) respond only to DAG. In contrast, atypical PKCs (ζ and ι/λ) are insensitive to both Ca^{2+} and DAG (*101*). Eye-PKC shares the highest sequence identity with human PKCα (52%) and PKCβ1 (53%). Previously, it was shown that a conventional PKC gains catalytic competence by undergoing three ordered phosphorylations (*101*). The first phosphorylation event is mediated by phosphoinositide-dependent kinase-1 (PDK-1), which phosphorylates the activation loop of PKC. This phosphorylation is a prerequisite

for the following two phosphorylation events that are contributed by an intramolecular autophosphorylation mechanism. It has been suggested that phosphorylation is involved in the maturation process of the enzyme, because 80% of PKC is already present in the triply-phosphorylated form in unstimulated cells (*101*).

Eye-PKC is a conventional PKC, however, little is known about how eye-PKC becomes catalytically competent. Here we sought to investigate the phosphorylation state of eye-PKC *in vivo* as a first step in understanding how eye-PKC gains its catalytic competence. By mass spectrometry, we show that eye-PKC is phosphorylated *in vivo* at Thr^{671}. We identified the spectra of doubly and triply charged phosphorylated peptides encompassing Thr^{671}. We also found the neutral ion losses for these peptides. Thr^{671} is an evolutionary conserved residue that corresponds to Thr^{638} and Thr^{643} of vertebrate PKCα and PKCβ1, respectively. Phosphorylation of Thr^{638} in PKCα and Thr^{643} in PKCβ1 has been shown previously to be important for attaining the catalytic competence of these PKCs. We also report that expression of catalytic competent eye-PKC in *Spodoptera frugiperda* (*Sf 9*) insect cells.

Materials and Methods

LC-MS analysis

LC-MS was performed as described before, by the Vanderbilt Mass Spectrometry Research Center. Approximately 10 pmoles of eye-PKC were excised from SDS/PAGE gels for in-gel digestion with either trypsin or chymotrypsin (*152*). The resulted peptides and data were analyzed and interpreted, as described before (see Chapter II).

Expression of GST-fusion proteins in bacteria

The region of INAD, containing the first two PDZ domains, and of TRP, containing residues 906-1275, were expressed as GST-fusion peptides in *E. coli* HMS174 cells, as described before (see Chapter II and III).

Expression of eye-PKC in insect cells

Expression of wild-type eye-PKC without or with a 6xHis tag (at either N- or C-termini) was employed in baculovirus *Sf9* cells (Invitrogen, La Jolla, CA). Transfection of *Sf9* cells with recombinant pVL1393 constructs was performed using the BaculoGold™ transfection kit (BD Biosciences, San Diego, CA). Transfected cells were incubated for 4 days at 27^0 C, and the supernatant containing the initial virus production (P1 virus) was collected and used to infect more cells to amplify the virus. A high titer virus stock solution was obtained after three rounds of amplification or three passages (P3 virus). *Sf9* cells were maintained as suspension cultures in TMN-FH medium (BD Biosciences) containing 10% fetal bovine serum (GibcoBRL, Grand Island, NY), 1% L-Glutamine (Sigma, Saint Louis, MO) and 0.1% Pluturonic acid (Sigma). To express eye-PKC, cells were grown to 2 x 10^6 cells/ml in 400 ml suspension cultures and then infected with the virus. After incubation at 27^0 C for two days, the cells were harvested by centrifugation at 3,000g for 15 minutes, and the pellet was kept at -80^0 C for later use. The extract was made by resuspending the pellet in a buffer containing 50 mM Phosphate buffer, 50 mM KCl, 5 mM β-mercaptoethanol, 1% triton X-100, and a cocktail of protease inhibitors, homogenized in a hand-held homogenizer chilled on ice, and

incubated on a rocker, for 1 hr at 4^{o}C. Then the extract was centrifuged at 3, 000g and 4^{o}C for 30 min, and the supernatant was used for the pull-down assay.

Purification of 6xHis eye-PKC

The *Sf9* cell lysates containing 6xHis tagged eye-PKC were incubated with Ni-NTA Agarose beads for 1 hr at 4^{o}C, on a rocking platform. Following a brief centrifugation, the supernatant was removed and the Ni-NTA Agarose beads containing the associated protein were washed five times with a buffer containing 50 mM Na-phosphate, 300 mM NaCl, and 10% glycerol, pH 8.0. Then, the recombinant eye-PKC was eluted from beads using a buffer containing 50 mM Na-phosphate, 300 mM NaCl, 10% glycerol, pH 6.0, and imidazole 0.5M. Purification of eye-PKC was assessed by SDS-PAGE and WB.

Peptide phosphorylation

Peptide phosphorylation was performed according to a method described before (*153*). Briefly, the synthetic peptide RRGRTGRGRRGIFR (EMD Biosciences, San Diego, CA) was incubated with either recombinant human PKCα or 6xHis tagged eye-PKC, and [γ-^{32}P]-ATP for 10 minutes, in the presence of PMA (1μM). The probes were subjected to 20% SDS-PAGE. The incorporation of radioactivity was detected by exposing the gel to a film.

Pull-down assay

25 μl of immobilized Glutathione-agarose beads were washed with 1X PBS and incubated with ten μg of GST-INAD or GST-TRP$^{906\text{-}1275}$ fusion proteins, or GST alone,

for 2 h at 4°C. After a brief centrifugation, beads were washed three times with extraction buffer or EB (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100 and a mixture of protease inhibitors), and incubated with 500 µl *Sf9* insect cell extracts expressing eye-PKC, for 1 hour at 4°C. After incubation, beads bound to GST fusion proteins and associated proteins (*i.e.* eye-PKC) were recovered and washed three times with EB and once with kinase reaction buffer (RB) (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.1 mM DTT, 0.4 mM EGTA, 0.7 mM $CaCl_{2)}$. The presence of pulled-down eye-PKC was analyzed by Western blotting.

In vitro kinase assay

Immobilized GST-fusion proteins together with the pulled-down proteins from the *Sf9* cell extract, were incubated at 30°C with 50 µl RB containing PMA (1 µM), 3 µCi of carrier-free [γ-^{32}P]-ATP in the presence of 100 µM cold ATP. 2X SDS/PAGE loading buffer was added to terminate the kinase reactions. Samples were then subjected to SDS/PAGE (10%) followed by Western blotting or Coomassie Blue staining. Dried and stained gels were subjected to autoradiography to assess phosphorylation.

WB analysis

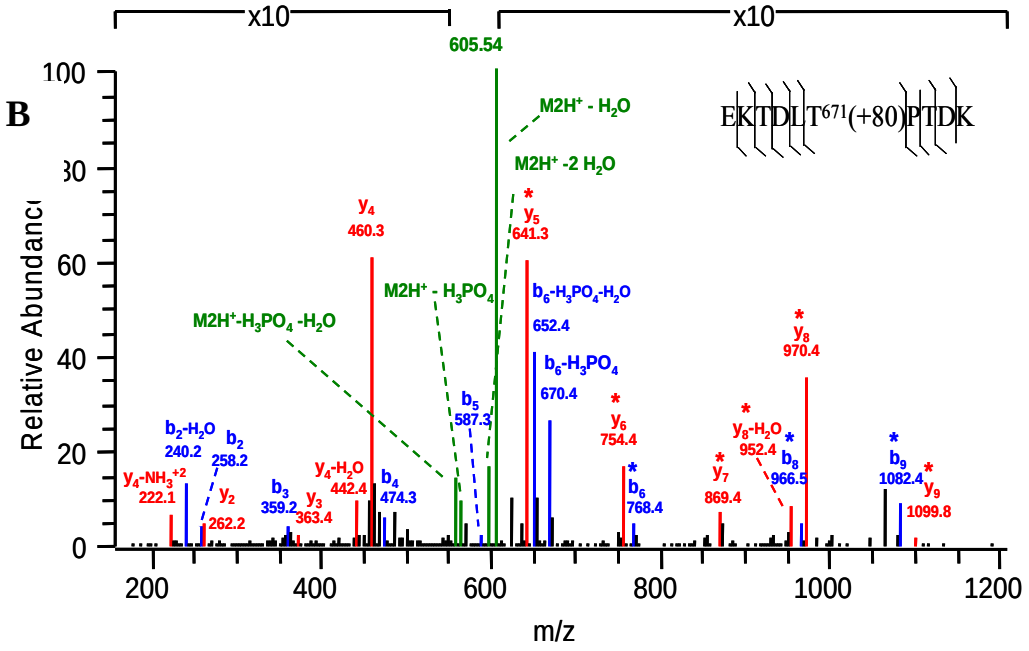
WB was performed as described before (see Chapter III).

Figure 15. Eye-PKC is phosphorylated *in vivo* at Thr^{671} as revealed by LC-MS analysis. (**A**) The sequence coverage of eye-PKC was approximately 48.3%. *Red* denotes tryptic and chymotryptic peptides identified by MS. (**B**) Low energy CID spectrum of the doubly charged phosphopeptide Glu^{667}-Lys^{676}. The spectrum was zoomed in 10-fold due to a very prominent neutral loss of ion. (**C**) Low energy CID spectrum of the doubly charged phosphopeptide Thr^{675}-Phe^{678}. This spectrum was also zoomed in 5-fold due to a prominent neutral loss of ion. The insets show the sequence of the phosphopeptide; *black lines* denote the identified cleavages. Fragment ions are labeled according to the accepted nomenclature. *b*-ions are coded *blue*, *y*-ions, *red*, and precursor ions, *green*. Spectra from the MS/MS/MS analysis of the neutral loss of phosphoric acid ions confirmed the sequence and the site of phosphorylation (data not shown).

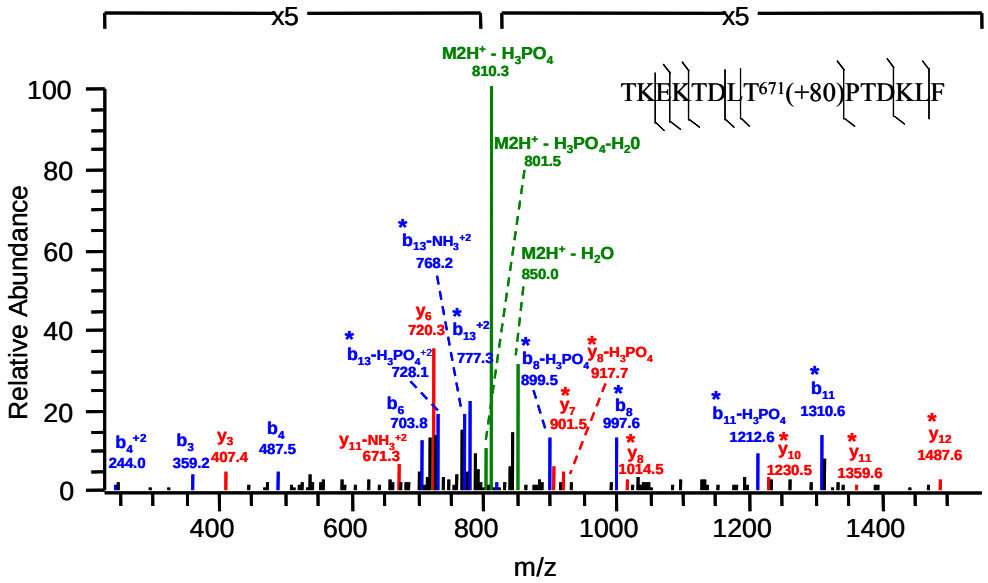
A

MAAAAVATPG ATVLPPSVPS AAPGAKAPAA GAGKGPGNLL EITGEANIVN YMKNRLRKGA MKRKGLEMVN GHRFGVRFFK NPTYCGHCKD
FIWGFGKQGF QCEECRFNIH QKCCKFVVFK CPGKDTDFDA DCAKVKHGWI STTYTTPTFC DECGLLLHGV AHQGVKCENC NLNVHHACQE
TVPPMCGADI SEVRGKLLLY VELKGNNLKV DIKEAANLIP MDTNGFSDPY IAVQMHPDRS GRTKKKTKTI QKNLNPVFNE TFTFELQPQD
RDKRLLIEVW DWDRTSRNDF MGSFSFSLEE LQKEPVDGWY KFLSQVEGEH YNIPCVDAFN DIARLRDEVR HDRRPNEKRR MDNKDMPHNM
SKRDMIRAAD FNFVKVIGKG SFGKVLLAER RGTDELYAVK VLRKDVIIQT DDMELPMNEK KILALSGRPP FLVSMHSCFQ TMDRLFFVME
YCKGGDLMYH MQQYGRFKES VAIFYAVEVA IALFFLHERD IIYRDLKLDN ILLDGEGHVK LVDFGLSKEG VTERQTTRTF CGTPNYMAPE
IVSYDPYSIA ADWWSFGVLL FEFMAGQAPF EGDDETTVFR NIKDKKAVFP KHFSVEAMDI ITSFLTKKPN NRLGAGRYAR QEITTHPFFR
NVDWDKAEAC EMEPPIKPMI KHRKDISNFD DAFTKEKTDL TPTDKLFMMN LDQNDFIGFS FMNPEFITII

B



C



Results

Eye-PKC is phosphoryated *in vivo* at Thr^{671}

To gain insight into how eye-PKC becomes catalytically competent, we explored the phosphorylation status of eye-PKC *in vivo*, using LC-MS analysis. To isolate eye-PKC from flies, we immunoprecipitated INAD complexes from light-adapted wild-type flies, using anti-INAD antibodies. The proteins in INAD complexes were separated by SDS-PAGE and visualized by Colloidal Blue staining. The 80 kDa protein band corresponding to eye-PKC was excised (Chapter II, Figure 9A), in-gel digested with trypsin or chymotrypsin, and the resulting peptide mixture was subjected to LC-MS. Fragments of peptides were analyzed and identified as previously described in Chapter II, by the SEQUEST algorithm (*140*), using a subset of *Drosophila* proteins from the non-redundant database from NCBI or Uniref100. The amino acid sequence coverage of eye-PKC was approximately 48% (Figure 15A). To examine the presence of phosphorylation sites in eye-PKC, we employed CID, which identified and confirmed the amino acid sequence of peptides EKTDLT^{671}PTDK and TKEKTDLT^{671}PTDKLF, and showed the presence of a phosphate group at Thr^{671} (Figures 15B and C). Moreover, the neutral loss of phosphoric acid was also detected for both peptides (data not shown), further confirming that Thr^{671} is phosphorylated. Together these data demonstrate that eye-PKC is phosphorylated *in vivo* at Thr^{671}.

Expression of eye-PKC in *Sf9* cells

To gain better insight into how eye-PKC is regulated, we expressed eye-PKC in *Sf9* cells, which support the post-translational modifications required for eye-PKC activity. Insect cells were infected with recombinant baculovirus containing full length *inaC* cDNA, and the expression of eye-PKC was assessed by Western blotting (Figure 16A). We show that the levels of eye-PKC reached a maximal level 48 hrs after the infection, but declined after 72 hrs (Figure 16B).

Once we established that eye-PKC is expressed in insect cells, we investigated whether the recombinant eye-PKC is catalytically competent. First, we purified the 6xHis tagged eye-PKC from insect cells by Ni^{2+}-agarose affinity chromatography (Figures 17A and B). Next, we tested whether this recombinant eye-PKC can phosphorylate a highly specific PKC peptide substrate, RRGRTGRGRRGIFR. As a positive control we used recombinant human PKCα. We found that PKCα phosphorylates the PKC substrate, whereas recombinant eye-PKC does not (Figure 17C). These results suggest that either the recombinant eye-PKC is not catalytically competent, or we are not using the right assay to test the catalytic competency of recombinant eye-PKC.

To avoid the impact of the His tag on eye-PKC function, we decided to express eye-PKC without any tag in *Sf9* cells, and investigate its catalytically competence. In *Drosophila*, eye-PKC, TRP and INAD form a macromolecular complex (*149*). Eye-PKC binds to the second PDZ domain of INAD (*54*), and has been shown to phosphorylate both INAD and TRP *in vitro* by immunocomplex kinase assays (*56, 75, 55*). In the complex kinase assay, phosphorylation of TRP by eye-PKC depends on the presence of INAD, which anchors eye-PKC in close proximity to TRP (see Chapter II).

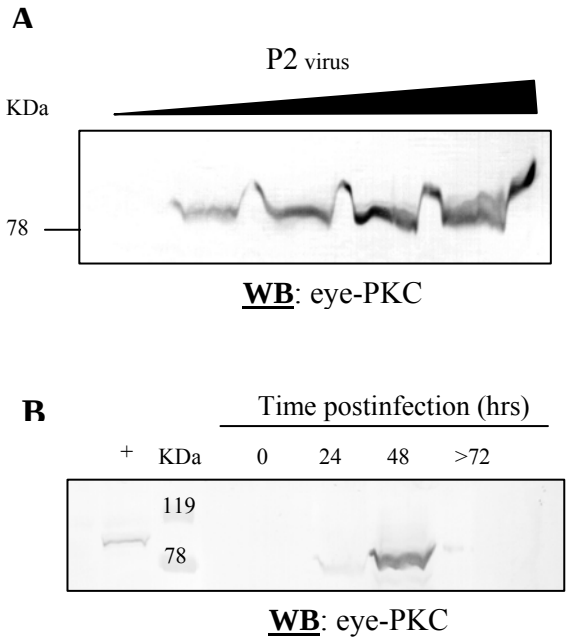


Figure 16. Generation of recombinant eye-PKC. (A) WB showing increasing expression of eye-PKC, following infection of insect cells with increasing amounts of P2 virus. (**B**) WB revealing that the maximum expression of eye-PKC is attained 48 hr postinfection. +, positive control consisting of wild-type fly head extract. WB, Western blotting.

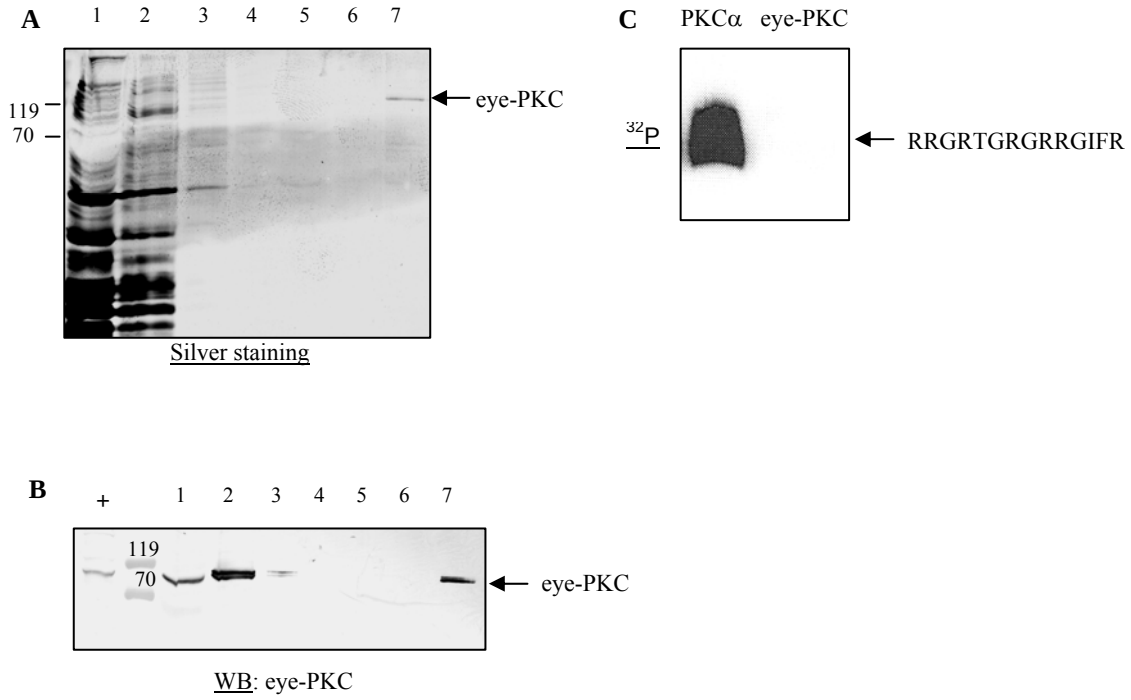


Figure 17. Recombinant eye-PKC does not phosphorylate a specific PKC substrate. **(A)** Silver stained SDS-PAGE gel showing purification of eye-PKC from insect cells. Purification was done as described in *Materials and Methods*. *Lane 1*, input, *lanes* 2, 3, 4, 5, 6 flow-through, *lane* 7, eluted eye-PKC. **(B)** WB following purification of eye-PKC. +, positive control (two fly heads), *lane 1*, input, *lanes* 2, 3, 4, 5, 6 flow-through, *lane* 7, eluted eye-PKC. WB, Western blotting. **(C)** Autoradiogram showing that recombinant human PKC phosphorylates a highly specific PKC substrate (RRGRTGRGRRGIFR), whereas recombinant eye-PKC does not.

To further investigate whether recombinant eye-PKC is catalytically competent, we employed the complex-dependent kinase assay using GST-fusion proteins containing either the first two PDZ domains of INAD or $TRP^{906-1275}$. Experimentally, immobilized GST-fusion proteins were incubated with *Sf9* insect cell extract expressing eye-PKC, and phosphorylation was monitored. We observed that GST-INAD became phosphorylated by *in vitro* kinase assay, since it was able to isolate eye-PKC from *Sf9* cell lysates, whereas no phosphorylation was detected upon incubation with extract prepared from uninfected *Sf9* cells (Figures 18A and B). In contrast, GST-$TRP^{906-1275}$ and GST were not phosphorylated following incubation with *Sf9* cell extract expressing eye-PKC, due to inability of these two proteins to recover eye-PKC from the extract (Figure 18B). Together these results demonstrate that the heterologously expressed eye-PKC is catalytically competent to phosphorylate INAD.

Discussion

An essential requirement for the catalytic competence of a PKC is its phosphorylation state (*151*). Studies on vertebrate PKCs have demonstrated that a PKC undergoes three ordered phosphorylations important for the enzyme maturation (*154*). The first phosphorylation, which is mediated by PDK-1, is the rate-limiting step, and occurs on a loop at the entrance to the catalytic site (activation loop) (*155*). Phosphorylation of the activation loop acts as a switch, and triggers the rapid autophosphorylation of the turn motif, which is a Pro-rich domain (*156*). Phosphorylation of the turn motif determines autophosphorylation of the hydrophobic site (*157*). The phosphorylation of the activation loop is required to initiate the autophosphorylation

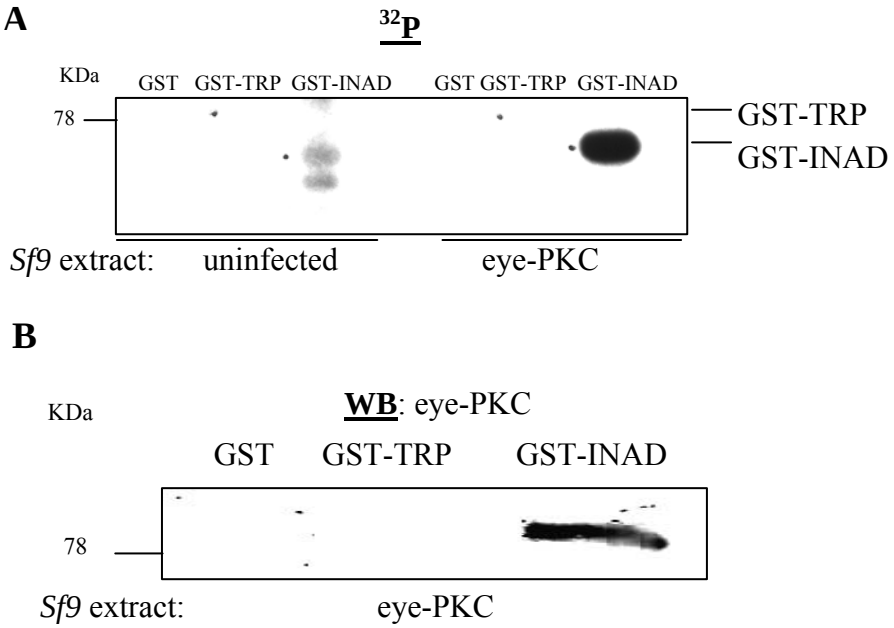


Figure 18. Recombinant eye-PKC is catalytically competent. (**A**) Autoradiogram indicating that INAD (PDZ1 and 2) is phosphorylated by recombinant eye-PKC. (**B**) GST-INAD pulls-down eye-PKC from infected *Sf9* cell extract, whereas GST and GST-TRP, do not. The protein molecular standards are denoted on the left.

events within the C-terminal sequence, however, once these events are completed, phosphorylation of the activation loop is not required for its activity (*158, 159*). Phosphorylation of the turn motif was shown to be both necessary and sufficient for the activity of a mature PKC (*143*), whereas phosphorylation at the hydrophobic motif affects only the stability of the enzyme, but not its function (*145*).

We report that eye-PKC is phosphorylated at Thr^{671} *in vivo*. Thr^{671} is located in the catalytic domain of eye-PKC. Sequence alignment of catalytic domains of *Drosophila* eye-PKC, *Calliphora* eye-PKC and vertebrates PKCα and βI discloses a high degree of conservation at Thr^{671}, suggesting that Thr^{671} is important for the eye-PKC function (Figure 19). Thr^{671} is the equivalent of Thr^{638} in PKCα and Thr^{642} in PKCβI. Both Thr^{638} and Thr^{642} have been indicated to be autophosphorylated, and are essential for these PKCs activity. Taken together, these data indicate that phosphorylation of Thr^{671} in eye-PKC may be sufficient for the activity of eye-PKC *in vivo*. Further studies are needed to support this hypothesis.

We also noticed a high degree of sequence conservation at Thr^{528} and Ser^{680} of eye-PKC respectively, with mammalian PKC. In mammalian PKC, these residues are known to be either phosphorylated by PDK-1, or autophosphorylated (Figure 19). However, the phosphorylation status of these two residues in eye-PKC *in vivo* is not known.

We also generated a catalytically competent recombinant eye-PKC in *Sf9* cells. The advantage of using insect cells to express a conventional PKC is due to the preservation of post-translational modifications that are required for attaining the catalytic competence of a PKC, as shown previously for recombinant rat PKCβII (*143*).

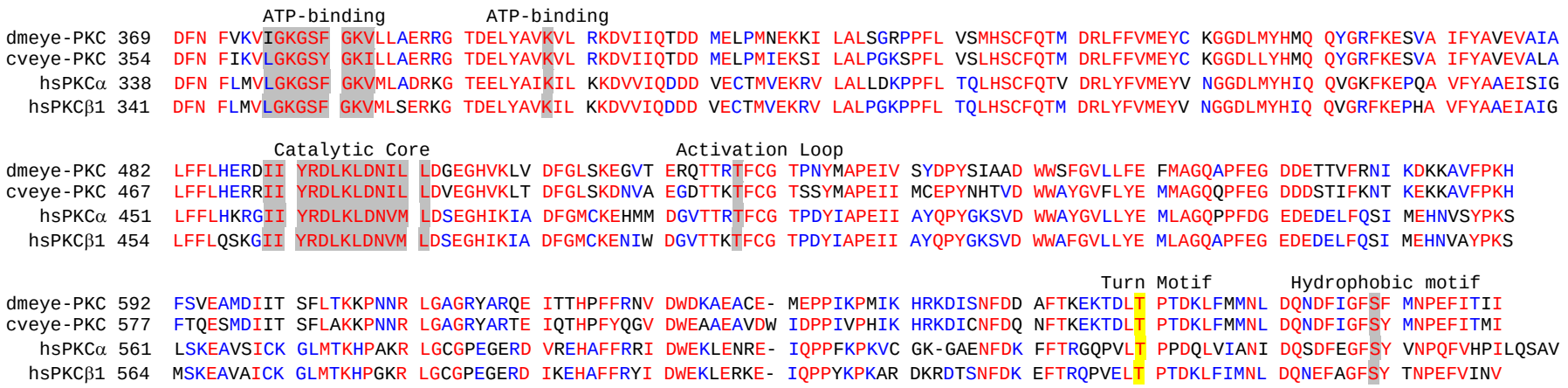
Initially, we expressed a His-tagged eye-PKC in *Sf9* cells, but this recombinant eye-PKC was not able to phosphorylate a highly specific PKC substrate. This may be because the His tag interferes with the proper folding or functional properties of the enzyme, or because we have not used the right assay. To avoid any interference of the His tag with the activity of eye-PKC, we generated a recombinant eye-PKC without a tag. Moreover, we employed a modified eye-PKC assay to address the kinase activity. In *Drosophila,* eye-PKC is anchored to the INAD complex by direct binding to INAD. INAD also interacts with NORPA and the TRP calcium channel. Here, we show that recombinant eye-PKC is able to bind and phosphorylate a fusion protein containing the first and second PDZ domains of INAD. In contrast, this recombinant eye-PKC does not bind and phosphorylate the fusion protein containing $TRP^{906\text{-}1275}$. This result is consistent with our previous data showing that phosphorylation of TRP requires INAD to anchor eye-PKC from fly head extracts (see Chapter II).

Summary and Future directions

In conclusion, here we report that eye-PKC is phosphorylated *in vivo* at Thr^{671}, a highly conserved autophosphorylation site that may play a role in maturation of eye-PKC. We also generated a catalytically competent eye-PKC, using the baculovirus infection of insect cells. To gain better insight into the regulation of eye-PKC by phosphorylation, more studies are needed. The availability of a heterologous expression system in which eye-PKC can be obtained and studied, is a first step in unveiling details regarding the regulation of eye-PKC. We also believe that generating and characterizing

functionally transgenic flies lacking the phosphorylation site at Thr^{671} may reveal important details regarding the regulation of eye-PKC *in vivo.*

Figure 19. Sequence alignment of catalytic domains of various PKCs. The three conserved phosphorylation sites known to be important for regulation of vertebrates PKCs are indicated in *gray* and *yellow*. Based on sequence homology, Thr^{671} of eye-PKC, shaded in *yellow,* is located within the turn motif of the enzyme. The ATP-binding domain, catalytic core, activation loop, turn and hydrophobic motifs are indicated. dmeye-PKC, *Drosophila melanogaster* eye-PKC, cveye-PKC, *Calliphora vicinia* eye-PKC, hsPKCα, *Homo sapiens* PKCα, hsPKCβ1, *Homo sapiens* PKCβ1. The alignment was done using MultAlin software (http://prodes.toulouse.inra.fr/multalin/multalin.html). Highly conserved residues are denoted in *red*, low conserved residues are denoted in *blue*.


ATP-binding
ATP-binding
dmeye-PKC 369 DFN FVKVIGKGSF GKVLLAERRG TDELYAVKVL RKDVIIQTDD MELPMNEKKI LALSGRPPFL VSMHSCFQTM DRLFFVMEYC KGGDLMYHMQ QYGRFKESVA IFYAVEVAIA
cveye-PKC 354 DFN FIKVLGKGSY GKILLAERRG TDELYAVKVL RKDVIIQTDD MELPMIEKSI LALPGKSPFL VSLHSCFQTM DRLFFVMEYC KGGDLLYHMQ QYGRFKESVA IFYAVEVALA
hsPKCα 338 DFN FLMVLGKGSF GKVMLADRKG TEELYAIKIL KKDVVIQDDD VECTMVEKRV LALLDKPPFL TQLHSCFQTV DRLYFVMEYV NGGDLMYHIQ QVGKFKEPQA VFYAAEISIG
hsPKCβ1 341 DFN FLMVLGKGSF GKVMLSERKG TDELYAVKIL KKDVVIQDDD VECTMVEKRV LALPGKPPFL TQLHSCFQTM DRLYFVMEYV NGGDLMYHIQ QVGRFKEPHA VFYAAEIAIG
Catalytic Core
Activation Loop
dmeye-PKC 482 LFFLHERDII YRDLKLDNIL LDGEGHVKLV DFGLSKEGVT ERQTTRTFCG TPNYMAPEIV SYDPYSIAAD WWSFGVLLFE FMAGQAPFEG DDETTVFRNI KDKKAVFPKH
cveye-PKC 467 LFFLHERRII YRDLKLDNIL LDVEGHVKLT DFGLSKDNVA EGDTTKTFCG TSSYMAPEII MCEPYNHTVD WWAYGVFLYE MMAGQQPFEG DDDSTIFKNT KEKKAVFPKH
hsPKCα 451 LFFLHKRGII YRDLKLDNVM LDSEGHIKIA DFGMCKEHMM DGVTTRTFCG TPDYIAPEII AYQPYGKSVD WWAYGVLLYE MLAGQPPFDG EDEDELFQSI MEHNVSYPKS
hsPKCβ1 454 LFFLQSKGII YRDLKLDNVM LDSEGHIKIA DFGMCKENIW DGVTTKTFCG TPDYIAPEII AYQPYGKSVD WWAFGVLLYE MLAGQAPFEG EDEDELFQSI MEHNVAYPKS
Turn Motif
Hydrophobic motif
dmeye-PKC 592 FSVEAMDIIT SFLTKKPNNR LGAGRYARQE ITTHPFFRNV DWDKAEACE- MEPPIKPMIK HRKDISNFDD AFTKEKTDLT PTDKLFMMNL DQNDFIGFSF MNPEFITII
cveye-PKC 577 FTQESMDIIT SFLAKKPNNR LGAGRYARTE IQTHPFYQGV DWEAAEAVDW IDPPIVPHIK HRKDICNFDQ NFTKEKTDLT PTDKLFMMNL DQNDFIGFSY MNPEFITMI
hsPKCα 561 LSKEAVSICK GLMTKHPAKR LGCGPEGERD VREHAFFRRI DWEKLENRE- IQPPFKPKVC GK-GAENFDK FFTRGQPVLT PPDQLVIANI DQSDFEGFSY VNPQFVHPILQSAV
hsPKCβ1 564 MSKEAVAICK GLMTKHPGKR LGCGPEGERD IKEHAFFRYI DWEKLERKE- IQPPYKPKAR DKRDTSNFDK EFTRQPVELT PTDKLFIMNL DQNEFAGFSY TNPEFVINV

CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

The wealth of information regarding the involvement of TRP channels in different physiological and pathological processes prompt the development of various strategies to intervene and study the function of TRP channels. One of these strategies is to study TRP in *Drosophila* (*13*). *Drosophila* is an attractive model system for understanding basic molecular mechanisms that underlie many biological processes. Particularly, *Drosophila* visual signaling is a G-protein coupled signaling pathway that shares similarities with the phototransduction pathway in vertebrates pRGCs. Mammalian pRGC detects irradiance (the environmental brightness) (*5*). Therefore, understanding *Drosophila* visual signaling can give us insight into not only how other TRP channels are regulated, but also the mechanism of pRGCs visual signaling.

In *Drosophila* photoreceptors, eye-PKC is crucial for deactivation of the light response (*53*). However, the molecular details remained elusive. This thesis describes the identification of an eye-PKC phosphorylation site in TRP, Ser^{982}, which is important for deactivation of the visual signaling. We show that transgenic flies lacking this phosphorylation sites exhibit defects in deactivation in response to bright light (see Chapter II). We also show that phosphorylation of the TRP channel by eye-PKC depends on the scaffolding protein INAD, which positions eye-PKC in close proximity to TRP (Chapter II). To further characterize how phosphorylation of TRP at Ser^{982} affects the TRP function, intracellular recordings can be employed. In addition, one can generate transgenic flies in which the phosphorylation state at Ser^{982} is mimicked by Asp (trp^{S982D})

substitution, and assess these flies electrophysiologically.

In Chapter III, we investigated another potential phosphorylation site at Ser^{884}. Our preliminary data indicate that Ser^{884} does not play a role in regulation of the light response, since transgenic flies lacking this phosphorylation site exhibit normal response to light stimulation. Expression of variable levels of TRP in these transgenic flies is intriguing, and awaits further investigation. This can be presumably addressed by exploring whether this is an age- or light-dependent process, or whether it is due to variable expression of the modified TRP. If this is an age- or light dependent process, phosphorylation at Ser^{884} may regulate the stability of TRP. The TRP-INAD interaction has been reported to be important for retaining TRP within rhabdomeres, and also for its protein stability (*149*). Therefore, to investigate whether a lack of phosphorylation at Ser^{884} in trp^{S884A} disrupts the interaction between INAD and TRP, immunoprecipitation studies can be employed.

Finally, in Chapter IV, we show that eye-PKC is phosphorylated *in vivo* at Thr^{671}. Thr^{671} is present within the turn motif of eye-PKC, and is evolutionary conserved. Thr^{671} corresponds to Thr^{638} and Thr^{643} of human PKCα and PKCβI, respectively, which have been indicated previously to be essential for attaining the catalytic competence of these two enzymes (*101, 154*). To investigate the functional significance of eye-PKC phosphorylation at Thr^{671}, one can generate transgenic flies either lacking this phosphorylation site, or mimicking the phosphorylation state at Thr^{671}, and characterize these flies biochemically and electrophysiologically. In Chapter IV we also report generation of a recombinant eye-PKC in *Sf9* cells. The availability of a heterologous

expression system in which eye-PKC can be obtained and studied will provide useful information concerning regulation of eye-PKC by phosphorylation.

In conclusion, we have identified an eye-PKC phosphorylation site in TRP, at Ser^{982}. Our work demonstrates that phosphorylation of Ser^{982} in TRP by eye-PKC is important for deactivation of the *Drosophila* visual signaling. We also found that eye-PKC is phosphorylated *in vivo* at Thr^{671}. The functional significance of phosphorylation of eye-PKC at this site requires further investigations.

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