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**Scaffolding Protein INAD Regulates Deactivation of Vision by Promoting Phosphorylation of Transient Receptor Potential by Eye Protein Kinase C in *Drosophila***

Daniela C. Popescu, Amy-Joan L. Ham, and Bih-Hwa Shieh

1Department of Pharmacology and Center for Molecular Neuroscience, Vanderbilt University Medical Center, and 2Department of Biochemistry and Mass Spectrometry Research Center, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

*Drosophila* visual signaling is one of the fastest G-protein-coupled transduction cascades, because effector and modulatory proteins are organized into a macromolecular complex (“transducisome”). Assembly of the complex is orchestrated by inactivation no afterpotential D (INAD), which colocalizes the transient receptor potential (TRP) Ca\(^{2+}\) channel, phospholipase C\(\beta\), and eye protein kinase C (eye-PKC), for more efficient signal transduction. Eye-PKC is critical for deactivation of vision. Moreover, deactivation is regulated by the interaction between INAD and TRP, because abrogation of this interaction in *InaD*\(^{p215}\) results in slow deactivation similar to that of *inaC*\(^{p209}\) lacking eye-PKC. To elucidate the mechanisms whereby eye-PKC modulates deactivation, here we demonstrate that eye-PKC, via tethering to INAD, phosphorylates TRP in vitro. We reveal that Ser\(^{982}\) of TRP is phosphorylated by eye-PKC in vitro and, importantly, in the fly eye, as shown by mass spectrometry. Furthermore, transgenic expression of modified TRP bearing an Ala substitution leads to slow deactivation of the visual response similar to that of *InaD*\(^{p215}\). These results suggest that the INAD macromolecular complex plays an essential role in termination of the light response by promoting efficient phosphorylation at Ser\(^{982}\) of TRP for fast deactivation of the visual signaling.

**Key words:** *Drosophila*; INAD; PKC; TRP; vision; phosphorylation

**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 system. *Drosophila* visual signaling is initiated with the activation of rhodopsin by light. Activated rhodopsin, via a Gq heterotrimeric protein, stimulates phospholipase C\(\beta\) (PLC\(\beta\)) named no-receptor potential A (NORPA) (Bloomquist et al., 1988). NORPA hydrolyzes PIP\(_2\) (phosphatidylinositol 4,5-bisphosphate) to inositol 1,4,5-trisphosphate (IP\(_3\)) and 1,2-diacylglycerol (DAG), which leads to opening of the transient receptor potential (TRP) Ca\(^{2+}\) and TRP-like channels, and depolarization of photoreceptors (Niemyer et al., 1996; Reuss et al., 1997). The key second messenger that activates the TRP Ca\(^{2+}\) channel is thought to be either DAG or its lipid metabolites (Chyb et al., 1999; Raghu et al., 2000a), whereas IP\(_3\) does not appear to play a role (Montell, 1999; Raghu et al., 2000b). DAG may have a dual function, because it also activates the eye-specific protein kinase C (eye-PKC) essential for deactivation of the light response (Ranganathan et al., 1991; Smith et al., 1991).

*Drosophila* visual signaling is one of the fastest G-protein-coupled transduction cascades (Zuker, 1996). The fast kinetics of vision is partly attributable to the formation of a macromolecular complex containing TRP Ca\(^{2+}\) channel (Huber et al., 1996b; Shieh and Zhu, 1996; Chevesich et al., 1997), NORPA (Shieh et al., 1997; Tsunoda et al., 1997; van Huizen et al., 1998), and eye-PKC (Tsunoda et al., 1997; Adamski et al., 1998; Kumar and Shieh, 2001). This complex is organized by inactivation no afterpotential D (INAD) (Shieh and Niemeyer, 1995), a scaffolding protein with five PDZ (postsynaptic density-95/disc-large/zonula occludens-1) domains. INAD regulates the subcellular localization and stability of these three proteins. Flies lacking INAD exhibit a profound reduction of the light response (Tsunoda et al., 1997).

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 (Shieh and Niemeyer, 1995; Henderson et al., 2000). In addition, the INAD–eye-PKC interaction is essential for the *in vivo* activity of eye-PKC, because expression of modified eye-PKC that does not interact with INAD, fails to rescue *inaC*\(^{p209}\) flies lacking eye-PKC (Adamski et al., 1998). Indeed, two proteins in the complex, INAD and TRP, were found to be phosphorylated *in vitro* by eye-PKC (Huber et al., 1996a, 1998; Liu et al., 2000).
Here, we report the identification and functional characterization of an eye-PKC phosphorylation site in TRP. We show that TRP is phosphorylated at Ser\textsuperscript{982} by eye-PKC and this phosphorylation depends on INAD \textit{in vitro}. By differential mass spectrometry (MS), we confirm that Ser\textsuperscript{982} of TRP is phosphorylated \textit{in vivo} by eye-PKC. Moreover, we demonstrate that transgenic flies lacking this phosphorylation site display a slow deactivation phenotype similar to that of \textit{inaD}\textsuperscript{−}\textsuperscript{−} strains. The asterisks indicate INAD degradation products. The protein standards (in kilodaltons) are denoted on the left.

\textbf{Materials and Methods}

Preparation of fly head extracts. Approximately 100 \mu l of young wild-type, \textit{inaD}\textsuperscript{−}\textsuperscript{−} 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 \times g), and the supernatant was used for the \textit{in vitro} complex-dependent kinase assay. Protein concentrations were determined by BCA (Pierce, Rockford, IL).

In vitro complex-dependent kinase assay. This assay consists of a glutathione S-transferase (GST) pull-down followed by an \textit{in vitro} kinase assay. GST fusion proteins were immobilized to glutathione-agarose beads and incubated with 500 \mu l of fly head extract (total protein concentration, 3.5–5 \mu g/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\textsubscript{2}, 5 mM 2-mercaptoethanol, 0.1 mM DTT, 0.4 mM EGTA, 0.7 mM CaCl\textsubscript{2}) and incubated at 30°C with 50 \mu l containing phosphor myristate acetate (PMA) (1 \mu M), 3 \muCi of carrier-free \([\gamma\textsuperscript{−32P}]\text{ATP}\) in the presence of 100 \muM cold ATP. SDS-PAGE loading buffer (2×) was added to terminate the kinase reactions. Samples were then subjected to SDS-PAGE analysis (6 or 10%) followed by Western blotting or Coomassie blue staining. Dried and stained gels were subjected to autoradiography or PhosphorImager analysis to quantify phosphorylation of fusion proteins. PMA, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole (Go6976), and recombinant human PK\textsubscript{Ca} were obtained from Calbiochem (San Diego, CA). \([\gamma\textsuperscript{−32P}]\text{ATP}\) (6000 Ci/mmol) was from PerkinElmer (Boston, MA). Glutathione-agarose beads were from Pierce. The synthetic peptide was purchased from Sigma-Genosys (Woodlands, TX).

\textbf{Liquid chromatography-MS analysis.} Liquid chromatography (LC)-MS was performed by the Proteomics Laboratory in the Vanderbilt Mass Spectrometry Research Center. Approximately 14 pmol of TRP was excised from SDS-PAGE gels for in-gel digestion with either trypsin or chymotrypsin (Ham, 2005). The resulting peptides were separated by reverse-phase HPLC 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 before analysis using the synthetic peptide Tp6K (AVAKGAGAR). Typical tune parameters were as follows: spray voltage of 1.8 kV, a capillary temperature of 150°C, a capillary voltage of 50 V, and tube lens of 100 V. 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 three 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 of SEQUEST algorithm (Yates et al., 1995), using a Drosophila subset of proteins from the nonredundant database from the National Center for Biotechnology Information (NCBI). 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 (Hansen et al., 2005). All possible modified peptides were verified by manual inspection of the spectra.

\textbf{P-element-mediated germline transformation.} Wild-type and modified \textit{trp} cDNA were subcloned into a modified pCaSpeR 4 vector (Thummel and Pirrotta, 1992) that contains \textit{Drosophila} hsp70 promoter without the hsp70 3′ trailer region. The P-element construct and a transposase plasmid (“wings-clipped”) were injected into y1[w67C23]/embryos (BRPC Transgenic Drosophila Core, Massachusetts General Hospital/Harvard Medical School, Charlestown, MA). Flies with the transgene integrated into the second or third chromosome were selected and made homozygotes in the \textit{trp}\textsuperscript{Pmod} background for additional analysis.

\textbf{Electroretinogram recordings.} Electroretinogram (ERG) recordings were performed using red-eye young flies (1–3 d of age) that were reared in a 12 h light/dark cycle. The flies were anesthetized by carbon dioxide (light intensity, 4.45 mW) was delivered by a fiber optic light source (Oriel, Stratford, CT) 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 (Molecular Devices, Sunnyvale, CA).

\textbf{Statistical analysis.} All bar graph data were analyzed with GraphPad (San Diego, CA) Prism 4.0 software one-way ANOVA. Data represent the means ± SEM, unless otherwise noted, from several independent experiments.

\textbf{Results}

\textbf{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 (Fig. 1A). Because the
C-terminal tail of TRP has been implicated in gating and regulation of the channel, phosphorilation 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 PKCa could phosphorylate these fusion proteins because both PKCa and eye-PKC belong to the conventional PKC family. Indeed, we found that TRP906–1275 containing the last 370 residues of TRP including the six putative PKC sites became phosphorylated by endogenous eye-PKC (Fig. 2A), whereas TRP906–1275 (lane 3, TRP906–1275, V1266D), which contains two PKC sites did not (data not shown). Sequences spanning TRP906–1275 failed to produce stable fusion proteins in *Escherichia coli* and therefore were not tested. The stoichiometry of TRP906–1275 phosphorylation by PKCa was ~0.55 pmol phosphate/mole fusion protein. These findings indicated that TRP906–1275 contains one PKC phosphorylation site.

**Phosphorylation of TRP906–1275 by eye-PKC is dependent on INAD in vitro**

Next we investigated whether TRP906–1275 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 TRP906–1275 were incubated with wild-type fly head extracts 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 TRP906–1275 pulled down INAD and eye-PKC, and became phosphorylated after the addition of a PKC activator, PMA, by this complex-dependent kinase assay (Fig. 2A, lane 2). To demonstrate that the observed phosphorylation of TRP906–1275 is dependent on INAD, we used fly extracts prepared from *inaC* and *inaD* mutants. *inaD* is a loss-of-function allele of *inaD* (Tsunoda et al., 1997), whereas *inaD* expresses a modified protein resulting in a loss of the TRP–INAD association (Shieh and Zhu, 1996). As shown in Figure 2A (lanes 1 and 2), both extracts failed to support TRP906–1275 phosphorylation by the complex-dependent kinase assay. In both cases, phosphorylation was diminished because TRP906–1275 was unable to isolate INAD and consequently, eye-PKC, from these two extracts (Fig. 2A, middle and bottom).

To further support the role of INAD in directing eye-PKC to TRP, we investigated phosphorylation of a modified TRP906–1275 containing an Asp substitution at Val1266, which has been previously shown to disrupt the interaction between TRP and INAD (Shieh and Zhu, 1996; Li and Montell, 2000). We found that phosphorylation of TRP906–1275, V1266D was greatly reduced (Fig. 2B, C), because this modified TRP failed to recruit INAD and, consequently, eye-PKC (Fig. 2B, bottom). Importantly, this modified TRP remained an excellent substrate for recombinant human PKCa (Fig. 2D). Together, these results indicate that the phosphorylation of TRP906–1275 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 TRP906–1275, we show that this phosphorylation is abolished in the presence of a specific conventional PKC inhibitor Go6976 (5 μM) (Fig. 3A). To further confirm that eye-PKC is responsible for the observed phosphorylation of TRP906–1275, we performed the complex-dependent kinase assay using extracts from *inaC* (Pak, 1979), which lacks endogenous eye-PKC (Smith et al., 1991). As expected, phosphorylation of TRP906–1275 and full-length INAD was greatly reduced, by 82 and 86%, respectively (Fig. 3B) (mean ± SEM; TRP906–1275, 17.77 ± 2.5%; INAD, 13.81 ± 4.13%; n = 3). The absence of phosphorylation is attributable to a lack of eye-PKC recovery when *inaC* extracts were used (Fig. 3B, middle). These findings indicate that eye-PKC is indeed responsible for phosphorylation of TRP906–1275.

**TRP is phosphorylated at Ser982 in vitro**

To investigate which of the six putative PKC sites in TRP906–1275 is phosphorylated by eye-PKC, we examined phosphorylation of two shorter TRP fusion proteins that contain one (TRP1157–1275) or four (TRP1030–1275) predicted PKC sites. We found that TRP1030–1275 and TRP1157–1275 displayed a drastic reduction of phosphorylation by 78 and 91%, respectively (Fig. 4A) (mean ± SEM; TRP1030–1275, 22.09 ± 7.78%; TRP1157–1275, 8.33 ± 5.43%; n = 3), by eye-PKC (Fig. 4B). Interestingly, these two fusion proteins were also not phosphorylated by recombinant PKCa.
sulted in a marked decrease of phosphorylation by PKCα, whereas a similar substitution at Ser958 did not (Fig. 4D) (mean ± SEM; TRP906–1275, 100%; TRP906–1275, S958A, 80.47 ± 22.24%; TRP906–1275, S982A, 29.46 ± 2.84%; n = 3). Consistently, fusion proteins containing both mutations (S958A and S982A) also exhibited a drastically reduced phosphorylation (Fig. 4D) (mean ± SEM; TRP906–1275, S958A, S982A, 18.88 ± 6.93%; n = 3).

To further confirm that Ser982 of TRP is a PKC phosphorylation site, we obtained a synthetic peptide, ALRAS982VKNVD, spanning Ser982 of TRP, and used it for in vitro kinase assays. This oligopeptide was a substrate of recombinant human PKCα with a $K_{i}$ of 263.1 μM and a $V_{\text{max}}$ of 17.35 pmol/min. Together, these data indicate that Ser982 represents a major in vitro PKC phosphorylation site in TRP906–1275.

TRP is phosphorylated in vivo at Ser982 by eye-PKC

Once we established that Ser982 of TRP was phosphorylated in vitro by eye-PKC, we investigated whether Ser982 is phosphorylated in vivo by eye-PKC using LC-MS analysis. First, we isolated the INAD complexes from light-adapted wild-type flies via immunoprecipitation, using anti-INAD antibodies. The proteins in the INAD complexes were separated by SDS-PAGE and visualized by staining with Coomassie Blue. The 145 kDa protein band corresponding to TRP was excised (Fig. 5A), 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 of SEQUEST (Yates et al., 1995), using a subset of Drosophila proteins from the NCBI database. We obtained ~70% amino acid coverage of TRP906–1275, S982A by eye-PKC using LC-MS analysis.

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 RAS982VKNVDEKSGADGKPGTM and revealed the presence of a phosphate group at Ser982. Moreover, we also found the spectra of the unmodified peptide as well as both the doubly and triply charged phosphopeptides (Fig. 5C,D). Importantly, only the unphosphorylated peptide RAS982VKNVDEKSGADGKPGTM was detected in TRP isolated from inacCp20 flies. Based on these data, we conclude that TRP is phosphorylated in vivo at Ser982 by eye-PKC.

trp982A displays slow deactivation of the visual response

To gain insight into the functional significance of TRP phosphorylation at Ser982, we generated and characterized transgenic flies expressing a modified trp, trp982A, in which the phosphorylation site is eliminated. As a control, we also generated transgenic flies expressing a wild-type trp (trpw1). 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 genetic background (trpP301) (Montell and Rubin, 1989; Shieh and Zhu, 1996). We first determined whether the modified TRP is stably expressed by Western blotting. Indeed, we observed that the TRP protein in trp982A flies reaches a steady-state concentration similar to that of wild-type flies [Oregon-R (OR)] or transgenic flies expressing a wild-type trp, trpw1 (Fig. 6A). It appears that basal transcription driven by the hsp70 promoter is sufficient for transcription of trp leading to wild-type level of TRP in trpw1 and trp982A 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 2 min, and then given a 2 s
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 (Fig. 6B). 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 (Fig. 6B). Remarkably, transgenic expression of trp^S982A rescued the trp^P301 phenotype but with delayed deactivation kinetics (Fig. 6B). Close inspection of the deactivation kinetics in ERG revealed two subcomponents: a fast and a slow component. The fast subcomponent occurs immediately after light termination and achieves over 50% repolarization. The fast subcomponent is followed by the slow subcomponent, which 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 for the brightest light stimulation (log I_0 = 0); the half-repolarization time of trp^S982A is approximately twofold longer than that of wild-type flies (Fig. 6C) (mean ± SEM; wild type, 0.80 ± 0.119 s; trp, 0.84 ± 0.064 s; trp^S982A, 1.66 ± 0.253 s; n = 5). In contrast, the amplitude of the ERG responses in trp^S982A was comparable with that of trp^wt flies (mean ± SEM; trp^wt, 18.008 ± 0.95 mV; trp^S982A, 20.71 ± 2.73 mV; n = 5), 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 attributable 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 twofold longer than that of wild type, regardless of the light intensity used (mean ± SEM; log I_0 = 0; inaC^P209, 2.216 ± 0.1 s; log 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_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_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). These results indicate that the deactivation defect in inaC^P209 is more complex than that of trp^S982A and suggest that phosphorylation of addi-
tional 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 \( \text{trp}^{S982A} \) in comparison with that of \( \text{InaD}^{p215} \) (Pak, 1979). \( \text{InaD}^{p215} \) contains a modified INAD, \( \text{INAD}^{\text{M442K}} \), which fails to associate with TRP (Shieh and Zhu, 1996). The lack of the TRP–INAD interaction leads to a slow recovery of the visual response (Shieh and Zhu, 1996). We found that \( \text{InaD}^{p215} \) displayed an ERG phenotype similar to that of \( \text{trp}^{S982A} \) with deactivation defects that manifested at bright light stimulation. Moreover, as for \( \text{trp}^{S982A} \), the deactivation kinetics of \( \text{InaD}^{p215} \) at low light intensities were indistinguishable from wild type (Fig. 6C) (mean ± SEM; half-repolarization time for \( \text{InaD}^{p215} \), \( \log I/I_0 = 0, 1.432 ± 0.064 \); \( \log I/I_0 = -1, 0.815 ± 0.045 \); \( \log I/I_0 = -2, 0.374 ± 0.052 \); \( \log I/I_0 = -3, 0.398 ± 0.041 \); \( n = 5 \)). These results indicate that the slow recovery of \( \text{InaD}^{p215} \) may be attributable to a loss of eye-PKC phosphorylation in TRP.

Together, our biochemical and electrophysiological analyses demonstrate that phosphorylation of TRP at Ser982 by eye-PKC is important for the rapid deactivation of visual signaling in \( \text{Drosophila} \).

Discussion

Reversible phosphorylation modulates the dynamics of signal transduction by transiently altering activities of signaling proteins. Members of the conventional PKC family (Newton, 1995), which are activated by \( \text{Ca}^{2+} \) and DAG, are capable of phosphorylating a wide variety of protein substrates for temporal and spatial regulation of signaling processes (Violin and Newton, 2003). In \( \text{Drosophila} \), eye-PKC is involved in the negative regulation of visual signaling, because \( \text{inaC}^{p209} \) flies lacking eye-PKC display abnormal desensitization, slow deactivation, and defects in light adaptation (Smith et al., 1991; Hardie et al., 1993). Eye-PKC is anchored to a macromolecular complex by tethering to INAD (Tsunoda et al., 1997; Adamski et al., 1998). Interaction with INAD enhances the stability of eye-PKC as well as targets eye-PKC to the rhodomers of photoreceptors (Tsunoda et al., 2001), in which visual signaling occurs. Importantly, the \( \text{in vivo} \) function of eye-PKC is regulated by interaction with INAD. Previously, it was shown that eye-PKC phosphorylates TRP \( \text{in vitro} \) (Huber et al., 1998; Liu et al., 2000). In the present study, we investigated the molecular basis of TRP phosphorylation by eye-PKC.

To mimic eye-PKC phosphorylation of TRP \( \text{in vitro} \), we designed a complex-dependent kinase assay. We demonstrated that the \( \text{in vitro} \) 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 \( \text{InaD}^{p215} \) that expresses a modified INAD devoid of the TRP binding (Shieh and Zhu, 1996), are not able to promote TRP phosphorylation. Together, these findings indicate that INAD targets eye-PKC to its substrates, similar to RACK (receptor for activated C kinase) (Liu et al., 2000). By the complex-dependent kinase assay, we identified Ser982 of TRP as an eye-PKC phosphorylation site. Moreover, we analyzed TRP isolated from flies by LC-MS and found that Ser982 of TRP is indeed phosphorylated \( \text{in vivo} \) by eye-PKC, because phosphorylated peptides encompassing Ser982 of TRP were present in wild-type, but absent in \( \text{inaC}^{p209} \) flies.

Next, we investigated the \( \text{in vivo} \) functional contribution of phosphorylation by characterizing transgenic flies expressing a modified TRP bearing an Ala substitution at Ser982 (\( \text{trp}^{S982A} \)).

Remarkably, these transgenic flies displayed prolonged deactivation kinetics in response to bright light stimuli, indicating that phosphorylation of TRP at Ser982 by eye-PKC is involved in inactivation of TRP, leading to fast deactivation. A model of the TRP regulation by eye-PKC is proposed (Fig. 7). TRP is an integral part of the INAD complex and is opened by light. After light termination, the visual response is rapidly deactivated. Although molecular mechanisms underlying deactivation remain elusive, \( \text{Ca}^{2+} \) is known to play a vital role in response termination (Hardie, 1991; Ranganathan et al., 1991, 1994; Peretz et al., 1994). The increased intracellular \( \text{Ca}^{2+} \) (primarily mediated by TRP) and DAG activate eye-PKC, which, in turn, phosphorylates TRP at Ser982. Phosphorylation of TRP leads to a rapid inactivation of the channel on cessation of the light stimulation (Fig. 7B), without affecting the interaction between TRP and INAD (data not shown). How does phosphorylation influence the TRP channel activity? Ser982 is located within the Lys-Pro-rich region of TRP (Fig. 5B), which may function in TRP gating (Sinkins et al., 1996).

We speculate that phosphorylation at Ser982 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 (Dulhanty and Riordan, 1994). It is also possible that phosphorylation of TRP at Ser982 affects the interaction with some yet-unidentified proteins that 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 \( \text{inaC}^{p209} \) or \( \text{trp}^{S982A} \). We found that \( \text{inaC}^{p209} \) displays a more complex deactivation defect, whereas \( \text{trp}^{S982A} \) exhibits prolonged deactivation only in response to bright light. These findings suggest that, in addition to TRP, eye-PKC phosphorylates other substrates for efficient termination of the light response. Indeed, eye-PKC has been shown to phosphorylate INAD (Huber et al., 1996a; Liu et al., 2000), but the functional relevance of this phosphorylation is not known. Furthermore, Gu et al. (2005) reported that eye-PKC is required for the \( \text{Ca}^{2+} \)-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 \( \text{Ca}^{2+} \)-dependent inactivation of the light-induced current was unaltered in \textit{inaD} \textit{p215}. This finding suggests the existence of a parallel \( \text{Ca}^{2+} \)-dependent mechanism in \textit{inaC} \textit{p209} by which TRP is inactivated or of an upregulation of a \( \text{Ca}^{2+} \)-dependent mechanism that activates other kinases (Matsutomo et al., 1994) to compensate for the loss of eye-PKC in \textit{inaC} \textit{p209}.

Importantly, \textit{trp} \textit{S982A} displays slow deactivation kinetics similar to that of \textit{InaD} \textit{p215}. \textit{InaD} \textit{p215} was isolated by Pak (1979) based on the \textit{ina} (inactivation afterpotential) phenotype elicited by ERG. By whole-cell recordings, Shieh and Niemeyer (1995) showed that \textit{InaD} \textit{p215} exhibits slow deactivation kinetics. However, Tsunoda et al. (1997) reported a delay in latency of the quantum bump and proposed that activation was affected in the \textit{InaD} \textit{p215} mutant. To resolve this discrepancy, Henderson et al. (2000) reexamined the mutant and concluded that the primary defect in \textit{InaD} \textit{p215} is prolonged deactivation and not slow activation. \textit{InaD} \textit{p215} expresses \textit{INAD} \textit{M445K}, which fails to associate with TRP (Shieh and Niemeyer, 1995). How does a loss of \textit{INAD}–TRP interaction lead to abnormal deactivation of visual signaling? It is likely that the lack of the \textit{INAD}–TRP interaction prevents the recruitment of TRP to the \textit{INAD} complex and, consequently, eye-PKC-mediated regulation. Indeed, both \textit{trp} \textit{S982A} and \textit{InaD} \textit{p215} exhibit similar deactivation defects, indicating that the molecular basis underlying the slow deactivation defect in \textit{InaD} \textit{p215} is attributable to a lack of negative regulation of the TRP channel by eye-PKC. Together, these findings suggest that formation of the \textit{INAD} complex is essential for fast deactivation of the visual response by promoting phosphorylation of TRP by eye-PKC. Moreover, Ser \textit{982} may be the sole eye-PKC phosphorylation site in TRP, because \textit{trp} \textit{S982A} and \textit{InaD} \textit{p215} display similar deactivation defects. A loss of \textit{INAD}–TRP interaction was previously investigated in transgenic flies expressing modified TRP in which the \textit{INAD}-interacting domain was deleted (\textit{trp} \textit{A1722}). Li and Montell (2000) reported a reduced light response with normal deactivation kinetics in \textit{trp} \textit{A1722}. These authors proposed that the suppression of the delayed termination, which is attributable to a reduced eye-PKC level in \textit{trp} \textit{A1722} is probably masked by a concomitant decrease in TRP and \textit{INAD} levels (Li and Montell, 2000).

To date, many proteins related to \textit{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 TRPL). (Ramsey et al., 2006). \textit{Drosophila} TRP belongs to the TRPC subfamily. Members of the TRPC subfamily are also activated by receptor-induced activation of phospholipase C (Montell et al., 2002) 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 TRPC3 was shown to contribute to its 

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