Interaction of Eye Protein Kinase C and INAD in Drosophila

LOCALIZATION OF BINDING DOMAINS AND ELECTROPHYSIOLOGICAL CHARACTERIZATION OF A LOSS OF ASSOCIATION IN TRANSGENIC FLIES

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Frances Mary Adamski, Mei-Ying Zhu, Frohar Bahiraei, and Bib-Hwa Shieh‡
From the Department of Pharmacology and Center for Molecular Neuroscience, Vanderbilt University, Nashville, Tennessee 37232-6800

Drosophila eye-specific protein kinase C (eye-PKC) is involved in light adaptation and deactivation. eye-PKC, NORPA (phospholipase Cβ), and transient-receptor-potential (TRP) (calcium channel) are integral components of a signal transduction complex organized by INAD, a protein containing five PDZ domains. We previously demonstrated the direct association between the third PDZ domain of INAD with TRP in addition to the carboxy-terminal half of INAD with the last three residues of NORPA. In this work, the molecular interaction between eye-PKC and INAD is defined via the yeast two-hybrid and ligand overlay assays. We show that the second PDZ domain of INAD interacts with the last three residues in the carboxy-terminal tail of eye-PKC, Thr-Ile-Ile. The association between eye-PKC and INAD is disrupted by an amino acid substitution (Ile-700 to Asp) at the final residue of eye-PKC. In flies lacking endogenous eye-PKC (inaCp215), normal visual physiology is restored upon expression of wild-type eye-PKC, whereas the eye-PKCp1700D mutant is completely inactive. Flies homozygous for inaCp209 and InaDp215, a mutation that causes a loss of the INAD-TRP association, were generated. These double mutants display a more severe response inactivation than either of the single mutants.

Based on these findings, we conclude that the in vivo activity of eye-PKC depends on its association with INAD and that the sensitivity of photoreceptors is cooperatively regulated by the presence of both eye-PKC and TRP in the signaling complex.

In Drosophila, visual transduction is a G-protein-coupled phospholipase C-mediated process that leads to depolarization of photoreceptors (see Refs. 1–3 for reviews). In this signaling pathway, rhodopsins are activated by light and catalyze the GDP/GTP exchange in the heterotrimeric G-protein, Gq. The α-subunit of Gq interacts with the phospholipase Cβ, NORPA, that catalyzes the hydrolysis of phospholipids to generate inositol 1,4,5-trisphosphate and 1,2-diacylglycerol (4). After the activation of NORPA, two cation channels, TRP1 and TRPL (TRP-like), are opened, leading to membrane depolarization (5). The mechanisms by which these two channels are gated remain elusive. One hypothesis is that the TRP calcium channel is controlled by depletion of internal calcium stores initiated by inositol 1,4,5-trisphosphate binding to its receptor (6), similar to the store-operated channel present in nonexcitable cells (7). However, a recent report suggests that an inositol 1,4,5-trisphosphate receptor is not involved in visual signaling (8). In addition calcium release from internal stores has not been directly implicated in visual excitation (9, 10). Despite the uncertain role of inositol 1,4,5-trisphosphate in photoreceptors, diacylglycerol generated by NORPA is a potent activator of protein kinase C (PKC). eye-PKC encoded by the inaC locus is known to be involved in the negative feedback regulation of visual transduction in Drosophila photoreceptors (11, 12). The importance of PKC as a regulator of the visual response is shown by the defective visual electrophysiology of the inaC mutants missing the eye-specific isoform of PKC. The inaC mutants display slow deactivation of the light response and are unable to adapt to different light intensities (11). In addition, inaC photoreceptors undergo light-dependent retinal degeneration (12).

eye-PKC has been shown to be a component of a signal transduction complex by immunoprecipitation and affinity chromatography assays (13–16). This complex is organized by an adaptor protein, INAD, that also interacts with two key components of the visual cascade, TRP and NORPA (13–18). The formation of this signaling complex is crucial for a functional visual signaling process, as flies lacking INAD show mis-localization of eye-PKC, TRP, and NORPA and display a drastically reduced response to light (16). INAD appears to act as a scaffold protein that assembles with these signaling proteins into a multiprotein complex. Formation of this macromolecular complex may be essential for the fast kinetics of signal transduction in Drosophila photoreceptors (14, 16). Since the gating mechanism of TRP is not known, investigations on the protein-protein interactions in this complex may shed light on events leading to depolarization. We previously reported a point mutation in INAD that eliminates the TRP interaction and brings about a slow recovery of the visual response (17, 19). Thus the INAD/TRP interaction appears to regulate the TRP calcium channel (16, 17). We also investigated the functional role of the NORPA/INAD association by generating transgenic flies expressing a modified NORPA that lacks the INAD interaction. The norpA transgenic flies display a unique electoretinogram recordings (ERG) characterized by delayed activation and slow deactivation. This phenotype of slow kinetics is also observed in InaDp10 that contains a missense mutation in the fifth PDZ domain leading to a loss of the NORPA association (16).

In this report, we define the respective binding domains in...
eye-PKC and INAD and demonstrate the functional consequence of this interaction in vivo. Our findings suggest the essential role of the eye-PKC and INAD association in regulating visual transduction. Potential substrates for eye-PKC that are involved in deactivation and desensitization of the visual response are discussed.

**EXPERIMENTAL PROCEDURES**

**Molecular Biological Techniques**

Standard molecular biology methods were used in subcloning, polymerase chain reaction (PCR), and nucleotide sequencing according to published procedures (20). DNA sequencing was performed using the Sequenase 2.0 kit (Amersham Pharmacia Biotech). Site-directed mutagenesis was carried out by the PCR-based overlapping extension method.

The Yeast 2-Hybrid System

The yeast two hybrid system used in this work is detailed in Golemis et al. (21). The yeast strain used was EGY191 (MATa, trp1, his3, ura3, 2ops-LEU2); the LacZ reporter plasmid was pSH18–34; the LexA fusion plasmid was pEG202, and the activation domain fusion plasmid was pG4–5. Methods for yeast transformation, testing protein-protein interactions, and checking protein expression were from Golemis et al. (21). Antibodies used to assay the level of fusion protein expression were polyclonal rabbit anti-HA (Zymed Laboratories Inc.) and anti-LexA (Invitrogen).

eye-PKC Fusion Protein Constructs

**PKC Full-length**—Primers flanking codons 1–381 of inaC were used to amplify 5′ sequence by PCR. The primers included 5′ BglII/EcoRI restriction sites and 3′ BamHI/XhoI restriction sites. BglII/XhoI restriction fragment was cloned into BamHI-XhoI sites of pGEX-4T-1 (Amersham Pharmacia Biotech). Then the NsiI-XhoI restriction fragment from pPKC (pPKC is a full-length inaC generated by RT-PCR) was cloned in to recreate inaC in pGEX-4T-1. The EcoRI-BamHI (blunt, vector-encoded) restriction fragment from pPKC was cloned into EcoRI-XhoI sites of pGEX-4T-1 before the EcoRI-EcoRI restriction fragment from the pGEX-4T-1 PKC clone was ligated into the EcoRI site of this construct.

**PKC-18**—BamHI-XhoI restriction fragment was subcloned from PKC into the BamHI-BamHI restriction sites of pEG202.

**PKC-18 TID**—The BamHI-BamHI restriction fragment from pPKC (codon 700, ATT mutated to GAT) was subcloned into BamHI restriction sites of PKC-18.

**PKC-NH2**—BamHI-XhoI restriction fragment was deleted from PKC-18.

**PKC-18** (codons 485 to 577 of inaC) was cloned into pEG202. The mutant and vector-encoded) restriction fragment was deleted from this construct (pCR2.I5 622) was subcloned into the HI restriction sites of pEG202.

**PKC-18** was subcloned into pJG4–5. From this the PKC-XhoI restriction fragment was cloned into pG4–5.

**PKD1**—Codon Changes to Disrupt the First PDZ Domain—Codon 22, AAG (K) to TCG (S); Codon 29, GCC (G) to GAA (E); Codon 30, ATC (I) to CAC (H); Codon 32, ATA (I) to ACA (T). Changes were introduced by two rounds of PCR amplification with two overlapping 5′ primers in conjunction with a 3′ primer. The SacII-XhoI restriction fragment was ligated into pCR2.I5, replacing wild-type sequence, then PDZ1 was constructed as per PDZ2.

**PDZ2**—Second PDZ Domain Codon Changes—Codon 254, AGG (R) to AGC (S); Codon 260, CTG (L) to CAG (Q); Codon 261, GGA (G) to GAA (E); Codon 262, CTC (L) to CAC (H). The resulting PCR product was cloned into PDZ1–2, replacing the wild-type BamHI-BamHI sequence. The PvuI site was deleted from EcoRI-XhoI sites of pBamHI-BamHI (PKC-18) (Stratagene), then the EcoRI-XhoI restriction fragment from this construct was subcloned into pJG4–5.

**PDZ4A**—A PCR product encompassing codons 485 to 577 of InaD with 5′ EcoRI and 3′ XhoI sites was subcloned into pG4–5.

**PDZ4B**—The EcoRI-PvuI site was subcloned into pG4–5 was subcloned into EcoRI-XhoI sites of pEG202.

**PDZ4C**—The BamHI-PvuI restriction fragment (codons 548–622) was subcloned into the BamHI-XhoI sites of pEG202. The EcoRI-XhoI restriction fragment was then subcloned into pJG4–5.

**PCR fragments and sequences across the junction of fused proteins were verified by DNA sequencing. Unless otherwise indicated, duplicate constructs were isolated and tested in triplicate.**

Western Blotting, Ligand Overlay Assay, and Overexpression of Fusion Proteins in Bacteria

These three procedures were carried out as described previously (17).

**Genetic Crosses**

Fly stocks were maintained at 25 °C in a 12-h dark/12-h light cycle. Recombination between marked InaD and (inaC, px, sp) chromosomes was performed using standard techniques. If recombination occurs, double mutants will have spotted (sp) marker, but not plexus (px). Potential double mutants were further evaluated for the presence of the and mutations by biochemical methods.

**Element-mediated Germ-line Transformation**—The mutant and wild-type eye-PKC cDNA were subcloned into a modified pCaSpeR 4 vector (24) that contains the Drosophila hsp70 promoter. The P-element construct and a transposase plasmid were injected into the embryos to generate transgenic flies (25). Flies with the transgene integrated into the second or third chromosome were selected and made homozygote for further analysis.

**ERG**

ERG recordings were carried out as described (19). Experimentally, flies were anesthetized by carbon dioxide and immobilized. Glass electrodes were filled with physiological saline (0.7% NaCl). Light stimulation was delivered by a fiber optic light source (Oriel). Signals were amplified as means of a WPI Dam 60 preamplifier (WPI) and digitized using Superscope II (GW Instruments).

**RESULTS**

Use of the Yeast Two-hybrid System to Investigate the Association between eye-PKC and INAD—We employed the yeast two-hybrid method using theLexA system (21), with eye-PKC (Fig. 1A) and INAD fused with the LexA DNA binding domain and a transcription activation domain, respectively. A positive interaction between the two proteins will activate transcription of two reporter genes. Leu2 activation results in growth of yeast on plates lacking leucine, and LacZ gives colonies with a blue
interaction, we tested an amino terminus fragment (PKC-NH2, activity (Fig. 1 which contains residues 18–700) as shown by LacZ reporter the basis for the interaction with INAD. We initially found that amino acid sequences unique to this PKC (12) and may be interact with INAD (Fig. 1). Although the carboxyl terminus (PKC 562–700, Fig. 1) was shown not to contain amino acids of eye-PKC is shown.

**FIG. 1. Mapping the INAD-interacting sequence in eye-PKC.** A, regions of eye-PKC tested for interaction with INAD. Shown is a schematic representation of eye-PKC (700 amino acid residues) (12). eye-PKC contains a diacylglycerol/phorbol ester binding domain (amino acids 72–186), C2 domain (amino acids 206–295), and a protein kinase domain (amino acids 371–629), indicated as patterned boxes sequentially left to right. Regions of eye-PKC used to examine interaction with INAD are detailed below. Numbering indicates the amino acid residues flanking the region tested. The sequence of the carboxyl-terminal 20-amino acids of eye-PKC is shown. B, a summary of the reporter activities and fusion protein expression in the interaction assay against full-length INAD in the yeast (see Fig. 3, A and B).

color on plates containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (Xgal). We used the expression of LacZ as a means for quantification. As a negative control, SNF1, a yeast serine/threonine protein kinase fusion (23), was shown not to interact with INAD (Fig. 1B).

The amino and carboxyl terminus domains of eye-PKC contain amino acid sequences unique to this PKC (12) and may be the basis for the interaction with INAD. We initially found that INAD interacts with both full-length eye-PKC and PKC-18 (which contains residues 18–700) as shown by LacZ reporter activity (Fig. 1B). To assess the role of the amino terminus in interaction, we tested an amino terminus fragment (PKC-NH2, Fig. 1A) and found that it did not interact with INAD (Fig. 1B). Although the carboxyl terminus (PKC-562-700, Fig. 1A) was the obvious determinant for association (Fig. 1B), we attempted to identify the scope of interaction by deleting eye-PKC to within 70 (represented as PKC-621-700), 26 (PKC-673-700), 20, 10, 4, and finally 3 amino acid residues of the carboxyl terminus without a loss of association with INAD (Fig. 1).

The last three residues in the carboxyl-terminal tail of eye-PKC, Thr-Ile-Ile (TII), fit the general motif for a PDZ domain interaction. PDZ domains typically interact with the carboxyl-terminal tail of proteins that terminate (0 position) with a hydrophobic residue (such as Val, Ile, or an aromatic residue, Phe) and have Ser or Thr or Asp at the −2 position (26). Amino acid substitutions were made to determine the critical residues required for the association. The final amino acid residue, Ile, when changed to Val, slightly decreases the interaction with INAD when tested in a protein fusion that contains the last 10 amino acid residues of eye-PKC (Fig. 1B). In contrast, the Asp or Asn substitution markedly reduces the association (Fig. 1B).

We also examined the Asp substitution in the context of PKC-18 (PKC-18 TID). PKC-18 TID fails to associate with full-length INAD (Fig. 1B) nor does it interact with the second PDZ domain of INAD (results not shown).

**Mapping the eye-PKC-interacting Sequence in INAD—**INAD was first predicted to contain two PDZ domains (19), but further analysis suggests that there are five PDZ domains in the protein (16, 27, 28). These PDZ domains are named sequentially PDZ1 to PDZ5 in the text. The PDZ domains of INAD in *Drosophila* and *Calliphora*, the blowfly, (13) are aligned in Fig. 2A to show the similarity of each individual PDZ domain from these two dipteran species. Although these five regions share the general characteristics of PDZ domains, the individual sequences (PDZ1 to PDZ5) are distinct and divergent. This is consistent with the notion that they interact with different proteins.

To map the region of INAD that interacts with eye-PKC, a series of INAD constructs were fused to the transcription activation domain and tested against PKC-18. These INAD fusion proteins are named according to the PDZ domains they encompass (Fig. 2B). We show strong association between PKC-18 with PDZ1–2 and PDZ2 (Fig. 2C). We also observed a weak yet detectable interaction between PKC-18 and PDZ1. However, no eye-PKC association with either PDZ2–5 or PDZ2–3 was detected (Fig. 2C). These results, observed consistently in several independent experiments, indicate that eye-PKC interacts with PDZ1 and PDZ2, with PDZ2 showing a stronger interaction (See Fig. 3C). To account for the lack of association between eye-PKC and PDZ2–3 or PDZ2–5, which also contain PDZ2, we propose that PDZ3 may interfere with PDZ2 binding to eye-PKC. Consistent with this hypothesis, is the observation that a weaker interaction between full-length INAD and eye-PKC was detected.

A recent report suggests that eye-PKC interacts with PDZ4 (16). We therefore paid special attention to this region of the protein. Three additional constructs that encompass PDZ4 (Fig. 2B) were tested, including PDZ4A, which contains exactly the same region of INAD as was reported to interact with eye-PKC (16). All of these PDZ4 fusion proteins fail to associate with eye-PKC. The expression level of these fusion proteins is similar to or higher than that of PDZ1–2 and should be sufficient to allow detection of an interaction with PKC-18 (Fig. 2C).

**Mutational Analysis of PDZ1 and PDZ2 Interaction with eye-PKC—**To confirm that eye-PKC interacts with INAD via the PDZ domains, amino acid substitutions were introduced in the PDZ domain to modify the structure. It is known from the crystal structure of a peptide-PDZ domain complex that the PDZ domain binding to the amino-terminal hydrophobic residue is dependent on a "carboxylate binding loop" (29, 30). Residues in this loop of PDZ1–2 and PDZ2–3 are shown boxed in Fig. 2A. eye-PKC appears to show a weak interaction with PDZ1 (Fig. 2C and 3C). We mutated PDZ1 by changing Lys-22 to Ser, Gly-29 to Glu, Ile-30 to His, and Ile-32 to Thr in PDZ1*. These nonconserved substitutions are likely to bring about a change in the structure of PDZ1. These mutations eliminated the interaction between PDZ1 and eye-PKC (Fig. 2C and 3C). We
analyzed a modified PDZ2 (PDZ2*) that contains the following substitutions: Arg-254 to Ser, Leu-260 to Gln, Gly-261 to Glu, and Leu-262 to His. We observed a marked reduction of the interaction between the modified PDZ2 and PKC-18 (Fig. 2C and 3C). Taken together, our findings provide evidence for a strong interaction between the second PDZ domain of INAD and the last three residues in the carboxyl-terminal tail of eye-PKC.

Further Evidence of the Involvement of PDZ2 in the PKC Interaction—To further confirm the results obtained above, we employed ligand overlay assay. This method was used successfully to define the interaction between the third PDZ domain of INAD and TRP (17). PDZ2, PDZ1–2, and PDZ4 were radiolabeled and probed in filters containing retinal extracts and bacterial extracts containing either an eye-PKC fusion protein or T7 gene 10, respectively. Fig. 4 shows that both PDZ2 and PDZ1–2 display strong and specific association with retinal eye-PKC and PKC fusion protein. In contrast, PDZ4 recognizes neither retinal PKC nor PKC fusion. Interestingly, in PDZ1–2, the presence of PDZ1 somehow potentiates the interaction of PDZ2 with its targets, whereas PDZ1 alone only weakly associates with PKC (data not shown). These findings agree with those obtained by the yeast two-hybrid assay, providing further evidence that PDZ2 interacts with eye-PKC.

Electrophysiological Analysis of Transgenic Flies Expressing eye-PKC/INAD Association in Drosophila Photoreceptors—In a modified PDZ2 (PDZ2*) that contains the following substitutions: Arg-254 to Ser, Leu-260 to Gln, Gly-261 to Glu, and Leu-262 to His, we generated transgenic flies lacking the interaction and examined the visual electrophysiology. We investigated a point mutation in eye-PKC, eye-PKCI700D, that eliminates the interaction of the kinase with INAD (Fig. 1B) without the modifications in those domains required for the predicted kinase activity (12). Transgenic flies expressing eye-PKCI700D under the control of a Drosophila heat-inducible promoter (hsp70) were generated in an eye-PKC null (inaCp209) genetic background. After heat shock treatment, the expression of eye-PKCI700D was comparable with that of eye-PKC in wild-type flies (Fig. 5A). The function of eye-PKC I700D was analyzed by ERG (Fig. 5B), showing that eye-PKC I700D displays a phenotype indistinguishable from that of inaCp209 (Fig. 5B). By contrast, transgenic flies expressing wild-type eye-PKC rescue the defective visual physiology of inaCp209 (Fig. 5B). We conclude that disruption of the eye-PKC-INA D interaction in eye-PKC/INAD results in a complete loss of the eye-PKC activity in vivo.

Electrophysiological Analysis of Double Mutants—InaDp215 flies express a mutant INAD protein that does not interact with TRP (17). To determine how the phenotype of inaCp209 flies is modified in the absence of the INAD/TRP interaction, we generated inaCp209, InaDp215 double mutants. The lack of eye-PKC...
was confirmed by Western blotting, and the presence of mutant INAD was confirmed by a loss of TRP binding in an overlay assay (Fig. 6).

ERG recordings of the \textit{inaC}^{9209} \textit{InaD}^{215} double mutant were compared with those of wild-type and single mutant flies. In the experimental paradigm used, flies were dark-adapted for 90 s then given two 5-s pulses of the orange light with a 6-s interval. As shown in Fig. 7, wild-type flies respond to light with fast kinetics and show desensitization during the light pulse. Importantly, the response to a subsequent stimulation of the same light intensity is not inactivated in wild-type flies, such that a second light pulse triggers a response almost the same as the initial one. Neither of the single mutant flies (\textit{inaC}^{9209} or \textit{InaD}^{215}) shows response inactivation under the experimental conditions (Fig. 7). In contrast, the double mutants display a marked reduction in this response to a second pulse of light. The peak amplitude of the second light response for wild-type, \textit{inaC}^{9209}, and \textit{InaD}^{215} flies was approximately 90\% that of the first light response (wild-type, 91 $\pm$ 6.2\%; \textit{inaC}^{9209}, 90 $\pm$ 5.0\%; \textit{InaD}^{215}, 95 $\pm$ 5.1\%, \textit{n} = 5). For the \textit{inaC}^{9209} \textit{InaD}^{215} double mutant, the peak amplitude of the second response was greatly reduced (44\% $\pm$ 7.5\%, \textit{n} = 10) (Fig. 7).

Double mutants also show abnormal deactivation similar to \textit{inaC}. We measured the rate of deactivation as the time required for the light response to decay to 50\% upon cessation of the light pulse. For wild-type and \textit{InaD}^{215} flies, deactivation kinetics were very rapid (wild-type, 27.4 $\pm$ 3.0 ms; \textit{InaD}^{215}, 29.1 $\pm$ 2.7 ms, \textit{n} = 10). For the \textit{inaC}^{9209} \textit{InaD}^{215} double mutant, the rate of deactivation was at least 80-fold slower than wild type and is similar to that of \textit{inaC} flies (\textit{inaC}, 2372 $\pm$ 640 ms; \textit{inaC}, \textit{InaD}, 2430 $\pm$ 430 ms, \textit{n} = 10) (Fig. 7).
DISCUSSION

We used the yeast two-hybrid system to map the regions of eye-PKC and INAD that interact and to determine the type of interaction. We found that the carboxyl-terminal tail of eye-PKC associates with predominantly the second PDZ domain of INAD. A fusion protein containing only the last three carboxyl-terminal residues of eye-PKC, Thr-Ile-Ile, interacts with INAD. This terminal sequence is consistent with the tripeptide motif, (S/T/D)X(V/I/F) (X is any amino acid) that specifies the association with a PDZ domain (26). Substitution of the ultimate isoleucine with an aspartic acid or an asparagine disrupts the interaction with INAD.

To gain insight into the in vivo function that the eye-PKC-INAD interaction specifies, we generated transgenic flies expressing eye-PKC<sup>I700D</sup> that will lack the INAD association. Significantly, these transgenic flies in the <i>inaC</i><sup>p209</sup> genetic background display an ERG phenotype of abnormal deactivation and desensitization similar to that of <i>inaC</i><sup>p209</sup>. This indicates that eye-PKC<sup>I700D</sup> fails to rescue the mutant phenotype. Thus, a lack of PKC interaction with INAD results in a complete loss of in vivo eye-PKC activity. This may be due to mis-localization of this enzyme, in turn rendering eye-PKC unable to phosphorylate specific substrates involved in regulation of the visual response.

To investigate how eye-PKC and INAD regulate the visual response, we characterized the electrophysiology of a...
*inaC* and *InaD* double mutant. We found that the double mutants display a more severe response inactivation, whereas *inaC* and *InaD* flies show no inactivation phenotype under the same experimental conditions. Response inactivation may result from depletion of limiting factors such as internal messengers and/or precursors, leading to exhaustion of the excitatory process (11). It is also likely that inactivation is due to defects in re-setting the proteins to the resting state such as conversion of metarhodopsin to rhodopsin (31, 32). Our finding suggests that the presence of both eye-PKC and TRP in the complex cooperatively contributes to regulation of sensitivity of photoreceptors.

One potential function of the signaling complex is to ensure eye-PKC and TRP are in close proximity for efficient regulation of the light response. A simple interpretation of the interdependence of eye-PKC and TRP is that eye-PKC regulates an intermediate, also associated with the signaling complex, that acts on TRP. Alternatively, eye-PKC may directly regulate TRP or regulate another molecule(s) via TRP. Such a model for eye-PKC regulation via the signaling complex has been proposed (14), and our analysis of the *inaC,InaD* double mutant provides the supporting evidence. Our finding that the response inactivation is more pronounced in the double mutant suggests that this regulatory step is partially activated in the absence of eye-PKC. It is possible that compensatory mechanisms differ during the development of the single and double mutants. It is also possible that this activity is constitutive or is modulated by another effector(s) in addition to eye-PKC.

Candidate substrates for eye-PKC include TRP, as mentioned, NORPA, INAD, or another unidentified molecule(s) present in the signaling complex. There is no evidence that either NORPA or TRP are phosphorylated during the light response. Light-induced phosphorylation has been demonstrated *in vitro* for *Calliphora* INAD (13). Thus INAD is a possible substrate of eye-PKC. INAD contains no domains with an obvious catalytic function, and if phosphorylation of INAD is part of the eye-PKC regulatory process, it may achieve its effect by altering the protein binding properties of INAD. This could change the composition or function of molecules within the signaling complex.

Our yeast two-hybrid and ligand overlay results both indicate that predominantly the second PDZ domain of INAD associates with eye-PKC, whereas no interaction was detected with PDZ4. This result is different from a previous report in which interaction of eye-PKC with the fourth PDZ domain of INAD was detected by affinity chromatography (16). We tested a total of five constructs that contained the fourth PDZ domain without any indication of this interaction. These included a fusion protein that contained exactly the same region as was tested before (16). One possible explanation for these conflicting results is that the different assay systems are measuring different types of association between eye-PKC and INAD. INAD may bind and cluster eye-PKC to the signaling complex, and it can also act as a substrate for the kinase activity (13). We showed that amino acid substitutions made in the second PDZ domain of INAD disrupted the eye-PKC binding. None of these amino acid changes were near serine or threonine residues that are putative PKC phosphorylation sites. Furthermore, mutations in the carboxyl-terminal tail of PKC abolish its binding to the second PDZ domain. Thus the interaction we describe for eye-PKC/INAD is a typical carboxyl-terminal tail/PDZ domain association. The basis of the reported interaction with the fourth PDZ domain remains to be determined. Another provocative explanation could be that eye-PKC may bind different PDZ domains of INAD during different physiological conditions. For example, phosphorylation of INAD may change the relative affinity of the interaction in PDZ2 and PDZ4. Clarification of the role of these two eye-PKC/INAD interactions will require analysis of transgenic flies expressing modified *InaD* in which these PDZ domain are mutated.

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