TRP and the PDZ Protein, INAD, Form the Core Complex Required for Retention of the Signalplex in *Drosophila* Photoreceptor Cells

Hong-Sheng Li and Craig Montell

Department of Biological Chemistry and Department of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Abstract. The light response in *Drosophila* photoreceptor cells is mediated by a series of proteins that assemble into a macromolecular complex referred to as the signalplex. The central player in the signalplex is inactivation no afterpotential D (INAD), a protein consisting of a tandem array of five PDZ domains. At least seven proteins bind INAD, including the transient receptor potential (TRP) channel, which depends on INAD for localization to the phototransducing organelle, the rhabdomere. However, the determinants required for localization of INAD are not known. In this work, we showed that INAD was required for retention rather than targeting of TRP to the rhabdomeres. In addition, we demonstrated that TRP bound to INAD through the COOH terminus, and this interaction was required for localization of INAD. Other proteins that depend on INAD for localization, phospholipase C and protein kinase C, also mislocalized. However, elimination of any other member of the signalplex had no impact on the spatial distribution of INAD. A direct interaction between TRP and INAD did not appear to have a role in the photoresponse independent of localization of multiple signaling components. Rather, the primary function of the TRP/ INAD complex is to form the core unit required for localization of the signalplex to the rhabdomeres.

Key words: PDZ • *Drosophila* • TRP • INAD • phototransduction

Introduction

A longstanding view of G protein–coupled signaling cascades is that they function through random collisions among receptors, G proteins, and downstream effectors. However, such a concept of highly mobile signaling molecules is inconsistent with the specificity inherent in G protein signaling. During the last decade, there has been a growing body of evidence to dispute a random collision model and to suggest that the components in G protein cascades might, in fact, be tightly coupled (for reviews see Rodbell, 1992; Neubig, 1994). Such compartmentalization would have great potential for preventing of adventitious cross-talk, and could potentially contribute to the speed of signaling.

A clear demonstration of a G protein signaling cascade that is organized into a supramolecular signaling complex, signalplex, has emerged from analyses of *Drosophila* phototransduction (for reviews see Pak, 1995; Montell, 1999). *Drosophila* phototransduction is among the fastest of G protein–coupled cascades and is activated and terminated in tens of milliseconds (Ranganathan et al., 1991). The components that function in the light response are situated in the photoreceptor cells within a specialized microvillar-containing organelle referred to as the rhabdomere. Many of the proteins that are critical for *Drosophila* phototransduction are linked through interaction with the modular adaptor protein inactivation no afterpotential D (INAD;[1] Huber et al., 1996a; Shieh and Zhu, 1996; Chevesich et al., 1997; Tsunoda et al., 1997; Xu et al., 1998; Wes et al., 1999). INAD consists primarily of five ~90–amino acid protein interaction motifs called PDZ domains (Shieh and Niemeyer, 1995).

PDZ motifs occur in a large variety of proteins and bind to a diversity of signaling, cell adhesion, and cytoskeletal proteins (for reviews see Kim, 1997; Sheng and Wyszynski, 1997; Craven and Bredt, 1998; Dimitratos et al., 1999; Fanning and Anderson, 1999; Schillace and Scott, 1999). As a consequence of these interactions, PDZ-containing proteins nucleate macromolecular assemblies, and do so at specialized membrane structures such as synapses. In most cases, binding to PDZ domains is mediated through an S/T-X-V motif or hydrophobic or aromatic residues at the

Address correspondence to Craig Montell, Department of Biological Chemistry 408WBSB, The Johns Hopkins University School of Medicine, 725 No. Wolfe St., Baltimore, MD 21205. Tel.: (410) 955-1199. Fax: (410) 614-9573. E-mail: cmontell@jhmi.edu

[1]*Abbreviations used in this paper:* ERG, electroretinogram; GST, glutathione S-transterase; INAD, inactivation no afterpotential D; PKC, protein kinase C; PLC, phospholipase C; TRP, transient receptor potential.

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COOH termini of the binding partners (Kim et al., 1995; Kornau et al., 1995; Songyang et al., 1997; Daniels et al., 1998).

A minimum of seven proteins binds directly to INAD. These include rhodopsin (Chevesich et al., 1997; Xu et al., 1998), phospholipase C (PLC; Huber et al., 1996a; Chevesich et al., 1997), protein kinase C (PKC; Huber et al., 1996a; Xu et al., 1998), the NINAC myosin III (Wes et al., 1999), calmodulin (Chevesich et al., 1997; Xu et al., 1998), and two light-dependent cation channel subunits, transient receptor potential (TRP; Shieh and Zhu, 1996) and TRPL (Xu et al., 1998). Three of the binding proteins, TRP, PLC, and PKC, require interaction with INAD for localization in the rhabdomeres (Chevesich et al., 1997; Tsunoda et al., 1997). Mutation of the INAD binding sites in PLC (Shieh et al., 1997; van Huizen et al., 1998; Cook et al., 2000) and PKC (Adamski et al., 1998) causes defects in the photoresponse; however, the effects may be a consequence of instability and/or mislocalization of the proteins rather than a specific requirement for tethering these proteins to INAD for proper signaling. Nevertheless, the signalplex also appears to have a direct role in signaling, independent of protein localization, as disruption of the NINAC/INAD interaction causes a delay in termination without causing any concomitant alteration in the concentration or the spatial distribution of NINAC (Wes et al., 1999).

Despite extensive biochemical and genetic analyses of the signalplex, the determinants required in vivo for localization of INAD are not known. Candidate anchoring proteins include the light receptor (rhodopsin), ion channels (TRP and TRPL), and the cytoskeletal protein, NINAC. Here, we show that TRP and INAD share a reciprocal requirement for retention in the rhabdomeres. INAD was mislocalized in an age-dependent manner in strong *trp* alleles, or as a consequence of deleting the INAD binding site in TRP. In contrast to a previous report (Shieh and Zhu, 1996), our results indicated that the direct interaction between TRP and INAD was mediated by the COOH-terminal residues. Furthermore, the TRP/INAD interaction was also required for the spatial distribution of two additional proteins, PLC and PKC, that depend on INAD for localization. We propose that TRP and INAD form the core complex, which is critical for retention of the signalplex in the rhabdomeres.

Materials and Methods

Generation of Wild-type and Mutant GST-TRP Fusion Proteins

Wild-type TRP consists of 1275 amino acids (Montell and Rubin, 1989). The Δ1272 mutation is a deletion of the COOH-terminal four residues in TRP (1272–1275), and V1266D is a valine to aspartic acid substitution at residue 1266. To generate the Δ1272 and V1266D mutations in the glutathione *S*-transferase (GST)-TRP fusion proteins, we performed oligonucleotide-directed mutagenesis using a single-stranded TRP genomic DNA fragment (nucleotides 4172–6480) inserted between the EcoRV and EcoRI sites of pBluescript KS$^+$ (pBSgTRP^{EK}; Stratagene). The wild-type and mutated DNA fragments were amplified by PCR (nucleotides 6004–6108) and inserted between the EcoRI and XhoI sites of pGEX-5X-3 (Amersham Pharmacia Biotech) to create the short-tail fusions. The short wild-type (encoding TRP residues 1247–1275) and mutant constructs are referred to as pGEX-STRP, pGEX-STRP-Δ1272, and pGEX-STRP-V1266D. The constructs encoding the long-tail fusions were generated by transferring SmaI/SalI fragments (*trp* nucleotides 5356–6480) from pBSgTRP^{EK} into the pGEX-5X-2 vector (Amersham Pharmacia Biotech). The long wild-type (encoding TRP residues 1030–1275) and mutant constructs are referred to as pGEX-LTRP, pGEX-LTRP-Δ1272, and pGEX-LTRP-V1266D. The GST-TRP constructs, as well as GST-INAD (pGST-INAD vector; Xu et al., 1998), were transformed into *Escherichia coli DH5α* cells and the proteins were expressed as described (Amersham Pharmacia Biotech).

Pull-down Assays

Fusion proteins were prepared from 20 ml *E. coli* cells transformed with pGST-INAD or the wild-type or mutant pGEX-STRP or pGEX-LTRP constructs described above. After inducing expression of the fusion proteins for 4 h with isopropyl-β-D-thiogalactoside (IPTG), the cells were pelleted by centrifugation, resuspended in 2 ml PBSIT buffer (PBS containing Complete™ enzyme inhibitor cocktail [Boehringer] and 1% Triton X-100), and lysed by sonication. The lysates were rotated at 4°C for 30 min to further solubilize the proteins, and cellular debris was removed by centrifugation at 14,000 rpm. Fusion proteins were purified from the supernatants with glutathione beads (Amersham Pharmacia Biotech). To perform the pull-down assays, 1 μg GST-TRP fusion proteins or 4 μg GST was coupled to 30 μl glutathione beads and incubated with an equal amount of ^{35}S-INAD or extracts prepared from fly heads (10 fly head equivalents) in 0.5 ml PBSIT. [^{35}S]methionine-labeled INAD was synthesized in vitro by coupled transcription/translation using the TNT kit (Promega) and pBS-INAD as the template. Fly head extracts were prepared by homogenizing 50 fly heads in 0.5 ml PBSIT buffer followed by centrifugation at 14,000 rpm for 30 min to remove debris. After incubation with the probes (^{35}S-INAD or head extracts), the beads were pelleted by centrifugation and washed four times with PBSIT. The proteins were eluted from the beads with 20 mM glutathione, and the eluates were fractionated by SDS-PAGE. The gels containing ^{35}S-INAD were dried and exposed to X-ray film. The proteins in the gels containing extracts from fly heads were subjected to Western blot transfer, the polyvinylidene fluoride (PVDF) membrane was stained with anti-INAD antibodies, and signals were detected using an enhanced chemiluminescence (ECL) kit (NEN Life Science Products).

Stability of TRP and INAD in Photoreceptor Cells

To determine the stability of TRP and INAD in vivo, we performed a pulse-chase experiment using ^{35}S-methionine. 300 late second instar or early third instar w^{1118} larvae were washed with PBS and transferred to a bottle with a square Whatmann No. 3MM paper adhered to the bottom. After draining the residual PBS that was transferred with the larvae, 0.7 ml labeling mixture (0.1 mCi/ml ^{35}S-methionine [> 1000 Ci/mmol; ICN Biomedicals], 0.5 M sucrose, and 2 mM of each of the following cold amino acids: Ala, Arg, Asn, Gln, Gly, His, Ile, Leu, Lys, Phe, Ser, Thr, Tyr, Val) was dispensed onto the paper. Adult flies <12 h after eclosion were transferred in groups of 50 flies to individual vials containing normal fly food media consisting of cornmeal, molasses, and brewer's yeast. Fly heads were harvested 1, 3, 5, and 9 d later, and were homogenized in PBSIT as described above.

TRP and INAD were immunoprecipitated using protein A beads coupled to 3 μl anti-TRP and anti-INAD antibodies. The beads were prepared by blocking 30 μl protein A-Sepharose beads (Sigma-Aldrich) with 3% BSA in PBSIT, incubating with the antibodies in 300 μl PBSIT for 1 h, and washing two times in PBSIT. The IgG-coupled beads were added to extracts prepared from 50 fly heads, and the total volume was increased to 1 ml with PBSIT. The beads were rotated for 2 h at 4°C, pelleted, and washed four times in PBSIT. Proteins were eluted from the pelleted beads by addition of 40 μl SDS–sample buffer and fractionated by SDS-PAGE (17 fly head equivalents), and the ^{35}S-labeled proteins were detected using Kodak BioMax MR film and a Kodak BioMax TransScreen-LE (low energy) intensifying screen. The exposed films were scanned into a computer, and the mean darkness values of the protein bands were determined using the histogram function available in the Adobe Photoshop 5.0 program. After the background darkness levels were subtracted, the value obtained with each time point was divided by that of the corresponding of 1.5-d group to obtain the relative radioactivities.

Generation of $trp^{\Delta 1272}$ Transgenic Flies

A 6480-nucleotide genomic fragment has been shown previously to fully rescue *trp* (Montell et al., 1985; Montell and Rubin, 1989). To generate the

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genomic rescue construct missing the genomic region encoding residues 1272–1275, we used the Δ1272 deletion created in pBSgTRP^{EK} (see above). pBSgTRP^{EK} includes an EcoRV/KpnI fragment spanning nucleotides 4172–6480. The EcoRV/KpnI fragment was excised from pBSgTRP^{EK}-Δ1272 and introduced into pKgTRP^{EE}. pKgTRP^{EE} includes an EcoRI/EcoRV fragment (nucleotides 1–4171) of TRP genomic DNA inserted into the pHFK vector. The pHFK vector contains a gene for kanamycin selection and a polylinker flanked at both ends with NotI sites. The full-length *trp$^{\Delta 1272}$* genomic DNA was excised with NotI and introduced into NotI site of the *ry*$^+$ P-element transformation vector pDM30 (Mismer and Rubin, 1987). The pDM30-*trp$^{\Delta 1272}$* DNA was injected into *trp^{CM},ry* embryos to generate P[*trp$^{\Delta 1272}$*] transformant flies (referred to as *trp$^{\Delta 1272}$* in the text). To facilitate the immunostaining analyses, the *w^{1118}* mutation was introduced into the transgenic flies to eliminate the red screening pigment in the compound eye that causes autofluorescence.

Immunolocalizations

Hemisected fly heads were fixed with paraformaldehyde and embedded in LR White resin as described (Porter and Montell, 1993). Cross-sections (0.5 μM) of the compound eyes were cut through the distal region of the retina, which includes the R7 cells, and stained with primary antibodies (1:250) and FITC-labeled secondary antibodies (1:50) as described (Porter and Montell, 1993). The PKC antiserum was generated in a rabbit against a fusion protein consisting of GST linked to amino acids 3–217 of the eye-specific PKC encoded by the *inaC* locus. We had previously prepared antibodies to INAD (Wes et al., 1999), TRP (Chevesich et al., 1997), and NINAC p174 (ZLA; Porter et al., 1992) in rabbits. Anti-Rh1 is a mouse monoclonal antibody (De Couet and Tanimura, 1987).

To perform the electron microscopy, fly heads were hemisected, fixed for 2–3 h in 2% paraformaldehyde, 2% glutaraldehyde, and 0.1 M sodium cacodylate (pH 7.4), washed three times in 0.1 M sodium cacodylate, postfixed for 2 h in 1% osmium tetroxide and 0.1 M sodium cacodylate, and washed two times for 5 min each in H_2O. After dehydration in 50%, 70%, and 90% acetone, the heads were embedded in LR White resin. The tangential sections examined were cut at a depth of 35 μm from the surface of the eyes and viewed by transmission electron microscopy.

Electroretinogram Recordings

Electroretinogram (ERG) recordings were performed as described (Wes et al., 1999). In brief, two glass microelectrodes filled with Ringer's solution were inserted into small drops of electrode cream placed on the surface of the compound eye and the thorax. A Newport light projector (model 765) was used to stimulate the eye. The ERGs were amplified with a Warner Electrometer IE-210 and recorded with a MacLab/4s A/D converter and the Chart v3.4/s program. All flies were raised at 25°C and recorded at room temperature.

Results

Mislocalization of TRP in InaD^{P215} Is Age Dependent

The original allele of *inaD*, referred to as *InaD^{P215}* (Pak, 1979), contains a missense mutation in PDZ3 that reduces interaction with TRP (Shieh and Niemeyer, 1995; Shieh and Zhu, 1996). We have found previously that the spatial distribution of TRP is severely disrupted in the photoreceptor cells of 2–3-d-old *InaD^{P215}* mutant flies (Chevesich et al., 1997). However, these data did not discern whether the interaction of TRP with INAD was required for targeting and/or retention of TRP in the rhabdomeres. If the TRP/INAD association was required exclusively for targeting, then the spatial distribution of TRP would be expected to be disrupted to a similar extent in young and old *InaD^{P215}* flies. Alternatively, if TRP depended on INAD for retention, then mislocalization of TRP would most likely become more severe with age.

Drosophila compound eyes consist of ~800 ommatidia, each of which contains eight photoreceptor cells (reviewed in Montell, 1999). Each photoreceptor cell contains a microvillar organelle, the rhabdomere, which is the site for photoreception and phototransduction. Six of the photoreceptor cells, R1-6, extend the full depth of the retina, whereas R7 and R8 occupy the central distal and proximal regions of the ommatidia, respectively. Consequently, only seven photoreceptor cells are present in any given plane of section. The individual rhabdomeres are separated by the central matrix, an extracellular region into which much of the old microvillar membrane enters upon turnover of the rhabdomeres (Blest, 1988).

To address whether TRP is physically linked to INAD for targeting and/or retention, we examined the spatial distribution of TRP in photoreceptor cells from young and old flies. In wild-type flies, TRP was localized exclusively in the rhabdomeres regardless of their age (Fig. 1 A). However, the spatial distribution of TRP was altered in *InaD^{P215}* in an age-dependent manner. During the late pupal period, TRP was restricted to the rhabdomeres in both wild-type and *InaD^{P215}* photoreceptor cells (Fig. 1 A). In adult flies <1 d old, TRP was detected primarily in the rhabdomeres, whereas a lower but significant fraction was observed in the cell bodies (Fig. 1 A). By 7 d after eclosion, the spatial distribution of TRP was severely disrupted in *InaD^{P215}*. In these older flies, at least as much anti-TRP staining was found in the central matrix as was found in the rhabdomeres (Fig. 1 A). The pronounced mislocalization of TRP in the older *InaD^{P215}* flies was specific, as other signaling proteins that function in phototransduction, such as the Rh1 rhodopsin (Fig. 1 B; Chevesich et al., 1997) and the PLC (data not shown and Chevesich et al., 1997), were localized normally in *InaD^{P215}*.

The age-dependent mislocalization of TRP in *InaD^{P215}* suggested that interaction with INAD was required for retention of TRP in the rhabdomeres. Alternatively, if TRP had a short half-life and was continuously synthesized at a high rate in vivo, the mislocalization of TRP in older *InaD^{P215}* might reflect a requirement for INAD for targeting in older flies. To differentiate between these models, we considered whether TRP was long or short lived by performing pulse-chase experiments. TRP was labeled in vivo by feeding third instar larvae ^{35}S-methionine. Immediately upon eclosion, the adult flies were fed fly food media, which was devoid of the radioactive amino acid, for 1–9 d. We found that the level of TRP decreased only ~25% between days 1.5 and 9.5 (Fig. 2, A and C). Moreover, the total level of TRP was relatively constant between days 1.5 and 9.5 (Fig. 2 B). Thus, it appeared that TRP was long lived, and that a large proportion of the TRP protein in 5–9-d-old flies was synthesized in young flies. Consequently, the mislocalization of TRP in 7-d-old *InaD^{P215}* (Fig. 1 A) appeared to be due to a defect in retention rather than in targeting to the rhabdomeres.

Spatial Distribution of INAD Is Dependent on TRP

INAD is required for proper localization of TRP, PLC, and PKC in the rhabdomeres (Chevesich et al., 1997; Tsunoda et al., 1997). However, the question arises as to the mechanism controlling rhabdomere-specific localization of INAD. Candidate INAD-localizing proteins are members of the signalplex that bind directly to INAD.

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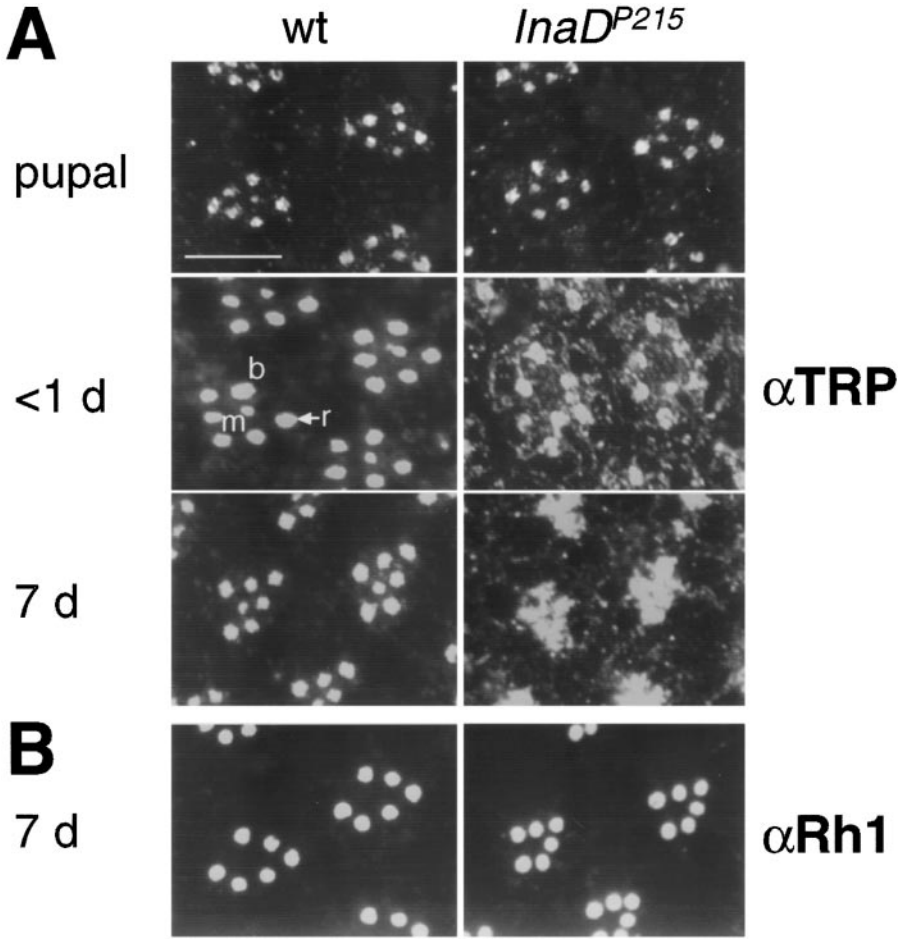
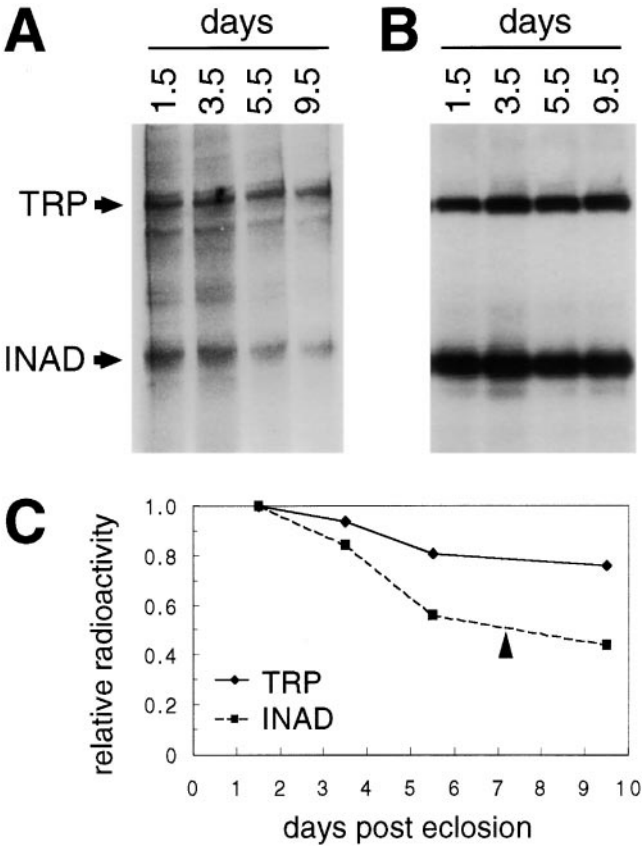


Figure 1. Spatial distribution of TRP and INAD in *InaD*^{P215}. Shown are tangential sections through the distal region of the compound eye (includes the six outer R1-6 cells and the smaller central R7). Each panel includes two entire ommatidia (30 × 22 μm) from either the late pupal period (pupal), young adults <1 d after eclosion (<1 day), or older adults 7 d after eclosion (7 days). Sections of wild-type (wt) and *InaD*^{P215} were stained with antibodies to (A) TRP (αTRP) and (B) Rh1 (αRh1). The αRh1 stains only six rhabdomeres, as it is specifically expressed in the R1-6 cells and not R7. Sections were subsequently stained with FITC-coupled secondary antibodies. The seven ovals seen in all of the wild-type and some of the *InaD*^{P215} ommatidia correspond to the rhabdomeres. The central matrix is the region in between the seven rhabdomeres of a single ommatidium. The cell bodies are located near the periphery of the ommatidia. r, rhabdomere; m, central matrix; b, cell body. Bar: 10 μm.



Thus, we examined the spatial distribution of INAD in mutants missing the individual INAD binding partners. No apparent defect was found in flies lacking PKC (*inaC* mutant), PLC (*norpA* mutant), TRPL (*trpl*), or Rh1 (*ninaE* mutant) (Fig. 3 A). In addition, we have found previously that the spatial distribution of INAD is not dependent on the putative molecular motor in the photoreceptor cells, the NINAC myosin III (Wes et al., 1999). However, a significant proportion of INAD was detected in the cell bodies of young *trp* mutants (Fig. 3 A). In old (7 d) *trp* flies, INAD was detected primarily in the cell bodies (Fig. 3 A).

Figure 2. Turnover of TRP and INAD in photoreceptor cells. TRP and INAD were labeled in vivo by feeding late second and third instar larvae ^{35}S-methionine and allowing the animals to complete development. Young flies (<12 h after eclosion) were transferred to normal fly food media for 1–9 d. TRP and INAD were immunoprecipitated with a cocktail of anti-TRP and anti-INAD antibodies, fractionated by SDS-PAGE, and exposed to film. (A) Autoradiography of immunoprecipitated TRP and INAD proteins collected 1, 3, 5, and 9 d after collecting the young flies (<12 h after eclosion). (B) Western blot showed total TRP and INAD protein levels in these samples. (C) Plot of relative levels of ^{35}S-labeled TRP and INAD at indicated times after eclosion. Results were obtained from the pixel intensities of the corresponding bands on the films exposed to autoradiography. The arrowhead indicates the time point in which the INAD signal declined by 50%.

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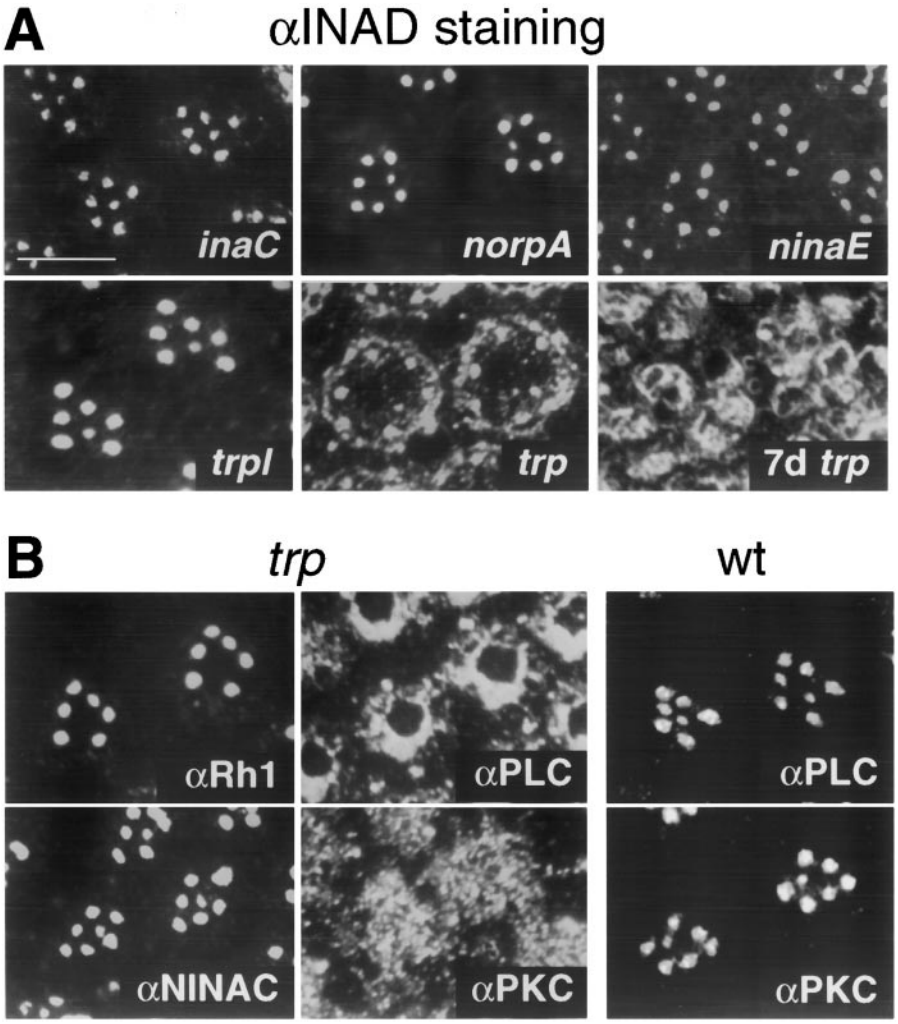

Figure 3. Mislocalization of INAD, PLC, and PKC in *trp* flies. With one exception, all cross-sections of compound eyes were obtained from adult flies <1 d old. The panel in the lower right corner of A was obtained from 7-d-old trp^{CM} mutant flies. (A) INAD localization in mutant flies. The protein eliminated in each mutant is indicated in parentheses: $inaC^{P209}$ (ePKC), $norpA^{P24}$ (PLC), $trpl^{302}$ (TRPL), $ninaE^{334}$ (Rh1), and trp^{CM} (TRP). α, anti-. (B) PLC and PKC, but not Rh1 or NINAC, are mislocalized in trp^{343} flies. The staining patterns of PKC and PLC in wild-type (wt) ommatidia are shown to the right. α, anti-. Bar, 10 μm.

PKC and PLC, but not Rh1 or NINAC, were also mislocalized in the cell bodies in young *trp* photoreceptor cells (Fig. 3 B). In addition, PKC staining was detected in the central matrix. These results were consistent with former reports that PKC and PLC require INAD for normal localization (Tsunoda et al., 1997), whereas Rh1 (Chevesich et al., 1997) and NINAC (Wes et al., 1999) do not. Thus, elimination of TRP resulted in the mislocalization of INAD, PLC, and PKC.

TRP Binds Directly to INAD through the COOH Terminus

Most PDZ-containing proteins bind to their targets via a COOH-terminal sequence that conforms to an S/T-X-V/I motif or that consists of aromatic and/or hydrophobic residues (Kim et al., 1995; Kornau et al., 1995; Songyang et al., 1997; Daniels et al., 1998). In the case of TRP, INAD was reported to bind to an internal S-X-V motif in the TRP tail (Shieh and Zhu, 1996). To confirm this observation, we constructed a V to D mutation in the S-X-V motif (V1266D; Fig. 4 A). In addition, we made a second construct in which the last four amino acids of TRP, SGWL, were deleted (Δ1272; Fig. 4 A). To test the effects of these mutations, we performed pull-down assays using GST-fusion proteins and a ^{35}S-labeled INAD probe. ^{35}S-labeled INAD bound to the GST fusion proteins consisting of the COOH-terminal 246 or 29 residues from wild-type TRP (referred to as long and short tails, respectively; Fig. 4, A and B). The short TRP fusion protein containing the V1266D mutation bound ^{35}S-labeled INAD as well as wild-type, although the V1266D mutation did decrease the interaction between INAD and the longer TRP tail. However, the Δ1272 mutation completely disrupted the interaction with INAD in the context of either the long or short TRP tail (Fig. 4 B). Thus, although there was some contribution of the internal S-X-V motif to the TRP/INAD interaction, the COOH-terminal four residues were essential for binding.

To exclude the possibility that posttranslational modifications of INAD might promote or interfere with binding to TRP, fly head extracts were also used in pull-down assays. INAD from wild-type fly head extracts bound to GST-TRP (Fig. 4 C). In addition, INAD also interacted with TRP^{V1266D}, although the amount of INAD that was pulled down was slightly reduced relative to that with wild-type GST-TRP (Fig. 4 C). Deletion of the last four amino acids from TRP obliterated the interaction with INAD (Fig. 4

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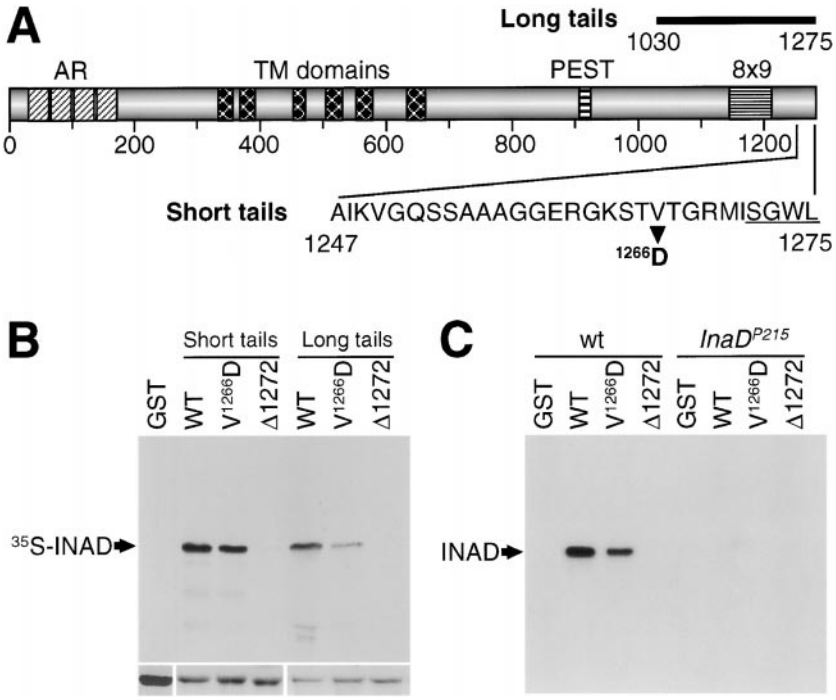

Figure 4. Pull-down assays indicated that the INAD binding motif was at the COOH terminus of TRP. (A) Schematic of TRP fragments fused to GST. Several domains in the 1275–amino acid TRP protein are indicated: AR, ankryin repeats; PEST, a putative degradation signal; TM, six putative transmembrane segments; and 8×9, eight residue motif (D-K-D-K-K-P-G/A-D) repeated in tandem nine times. Long or short portions of the COOH-terminal tail of TRP were expressed as GST fusion proteins. The wild-type long tail extended from residue 1030 to 1275, and the wild-type short tail included residues 1247–1275. The valine to aspartic acid substitution of residue 1266 (V1266D) and the last four residues in TRP (SGWL) that are deleted in Δ1272 are indicated in the sequence of the short tail. (B) Pull-down assay using GST-TRP fusion proteins bound to glutathione beads and an in vitro–translated ^{35}S-INAD probe. The following proteins were bound to beads: GST only, GST fused to wild-type short or long TRP tails (WT), and GST fused to short or long TRP tails harboring the V1266D mutation or missing the COOH-terminal four residues (Δ1272). The beads were incubated with the ^{35}S-INAD probe and washed, and the bound proteins were eluted with glutathione. The eluted proteins were fractionated by SDS-PAGE, and the concentration of probe that bound and eluted was detected by exposing the dried gel to film. The relative levels of GST and GST fusion proteins that bound and eluted from the beads (shown at the bottom) were determined by fractionating an equal volume of each eluate by SDS-PAGE and staining with Coomassie blue. The bands are shown as comigrating to conserve space. The size of the GST only, the GST short-tail fusions, and the GST long-tail fusions are 28, 31, and 56 kD, respectively. (C) INAD from wild-type and *InaD*^{P215} fly head extracts were used as probes in pull-down assays with GST fused to the short TRP tails. The beads were incubated with the extracts and washed, the eluted proteins were fractionated by SDS-PAGE, and a Western blot was probed with anti-INAD antibodies.

C). Identical results were obtained with either the short (Fig. 4 C) or long tails (data not shown). In parallel control experiments, all TRP tails failed to bind INAD from *InaD*^{P215} head extracts.

The missense mutation in PDZ3 of INAD^{P215} could potentially disrupt interactions with one or more proteins in addition to TRP. To determine the specific role of the TRP/INAD interaction, we generated transgenic flies, P[*trp*$^{\Delta 1272}$], expressing TRP channels missing the last four amino acids, which are crucial for binding to INAD. It has been shown previously that the TRP protein declines in an age-dependent manner in *InaD*^{P215} (Tsunoda et al., 1997). This effect was even more dramatic in *trp*$^{\Delta 1272}$. Based on Western blot analysis, the TRP protein level in <1-d-old *trp*$^{\Delta 1272}$ was comparable to that in wild-type flies (Fig. 5 A). In 7-d-old flies, the level of TRP decreased more than sixfold in *trp*$^{\Delta 1272}$ compared with a less than threefold reduction in *InaD*^{P215} (Fig. 5 A). The level of INAD protein did not decrease significantly in 7-d-old *trp*$^{\Delta 1272}$ flies; however, there was a slight age-dependent reduction in the *trp*^{343} flies (Fig. 5 B). The concentrations of other members of the signalplex, such as PLC and PKC, were also unchanged in 7-d-old *trp*$^{\Delta 1272}$ (Fig. 5 B). Thus, the mislocalization of INAD, PLC, and PKC in *trp*$^{\Delta 1272}$ did not appear to be a secondary effect of protein degradation. Moreover, these results demonstrate that mislocalization of signaling components from the rhabdomeres does not obligatorily result in an increased turnover of the proteins. The concentrations of INAD, PLC, and PKC were decreased in *trp*^{343} (Fig. 5 B). This effect appeared to be due to degeneration of the rhabdomeres, as the level of NINAC p174 was also reduced (Fig. 5 B), even though it is not dependent on interaction with the signalplex for stability or localization (Wes et al., 1999). To address whether the TRP$^{\Delta 1272}$ protein from transgenic flies could bind INAD, we performed pull-down assays using GST-INAD and extracts from wild-type and *trp*$^{\Delta 1272}$ fly heads. As expected, wild-type TRP but not TRP$^{\Delta 1272}$ from head extracts interacted directly with INAD (Fig. 5 C).

Age-dependent Mislocalization of TRP and INAD in trp$^{\Delta 1272}$ *Flies*

To determine the requirement for the TRP/INAD interaction for localization of TRP and INAD, we stained sections of *trp*$^{\Delta 1272}$ compound eyes with antibodies to each of the two proteins. During the first 24 h after eclosion, a large proportion of the TRP$^{\Delta 1272}$ staining was detected in the rhabdomeres (Fig. 6 A). In addition, significant staining was detected in the cell bodies. By 7 d after eclosion, TRP$^{\Delta 1272}$ was no longer concentrated in the rhabdomeres relative to the cell bodies (Fig. 6 A). In parallel with the mislocalization and reduction of TRP$^{\Delta 1272}$, INAD also displayed age-dependent alterations in spatial distribution in *trp*$^{\Delta 1272}$ flies. The localization of INAD was mildly disrupted in young *trp*$^{\Delta 1272}$ flies (Fig. 6 C), but was normal in

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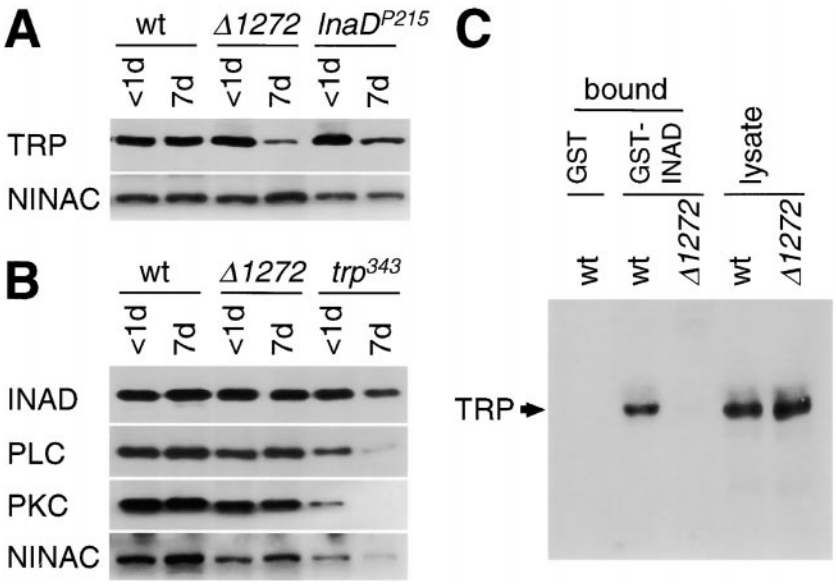

Figure 5. TRP/INAD interaction was disrupted in $trp^{\Delta 1272}$ transgenic flies. (A) Western blot comparing age-dependent changes in TRP protein levels in $trp^{\Delta 1272}$ and $InaD^{P215}$ flies. Extracts prepared from wild-type (wt), $trp^{\Delta 1272}$, and $InaD^{P215}$ flies (<1 d and 7 d old) were fractionated by SDS-PAGE, and a Western blot was probed with anti-TRP antibodies. NINAC p174 was probed with anti-NINAC antibodies as a control for protein levels. (B) Age-dependent changes in INAD protein levels in $trp^{\Delta 1272}$ and trp^{343} mutant flies. Shown is a Western blot probed with anti-INAD antibodies. The Western blot was also probed with anti-PLC, anti-PKC, and anti-NINAC p174 antibodies. (C) Pull-down assay performed with GST-INAD, bound to glutathione beads, and probed with head extracts from <1-d-old wild-type and $trp^{\Delta 1272}$ flies. The eluted proteins were fractionated by SDS-PAGE and probed with anti-TRP antibodies. Shown on the right is the concentration of TRP in the lysates (20% of input).

similarly aged *norpA* and *inaC* flies (Fig. 3 A). This mild mislocalization did not appear to be due to degeneration, as the morphology of $trp^{\Delta 1272}$ rhabdomeres was similar to that observed in wild-type, *norpA*, and *inaC* (Fig. 6 E). By 7 d after eclosion, the concentration of INAD in the rhabdomeres of $trp^{\Delta 1272}$ flies had declined dramatically (Fig. 6 C). Given that the half-life of INAD was ~5.5 d in vivo (Fig. 2, A and C) and that the proportion of INAD remaining in the rhabdomeres of 7-d-old $trp^{\Delta 1272}$ was barely detectable, it appeared that TRP was required for retention of INAD in the rhabdomeres.

The mislocalization of TRP and INAD in $trp^{\Delta 1272}$ flies was much less severe in flies maintained in the dark. In contrast to 7-d-old $trp^{\Delta 1272}$ flies reared under a normal light/dark cycle, a high proportion of TRP and INAD localized to the rhabdomeres in 7-d-old dark-reared flies

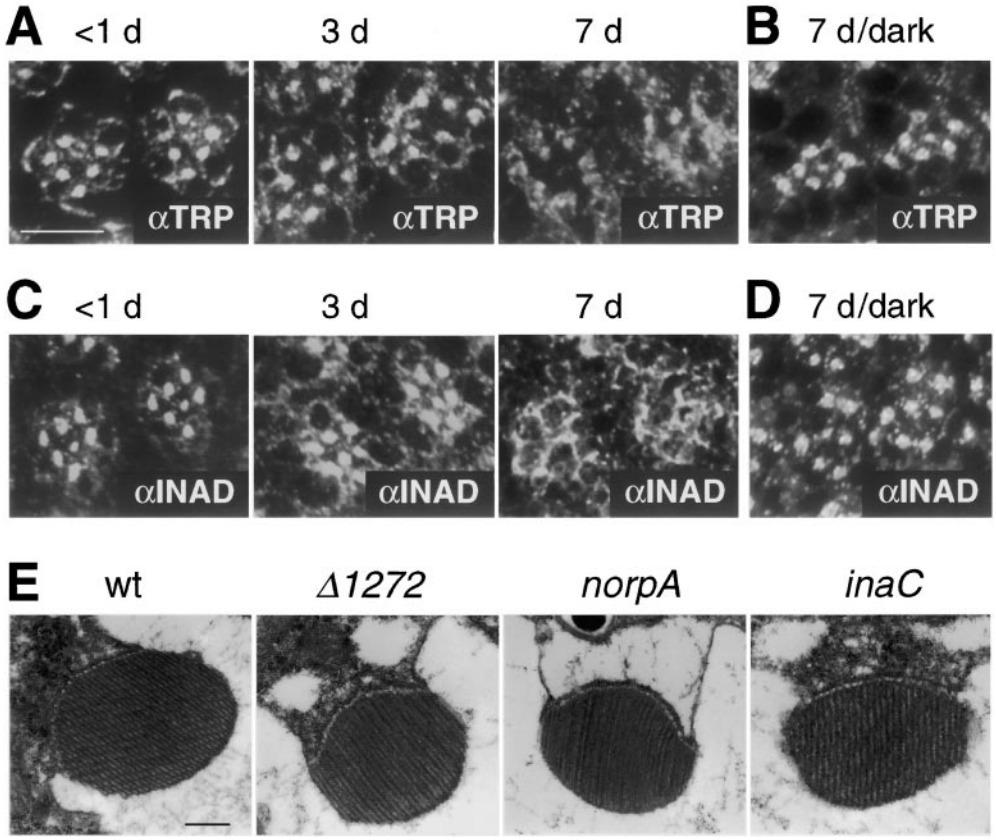

Figure 6. Spatial distribution of TRP and INAD in $trp^{\Delta 1272}$ flies. Tangential sections of adult compound eyes shown in A–D were obtained from $trp^{\Delta 1272}$ at the indicated ages (<1, 3, and 7 d after eclosion). (A) Sections were prepared from flies reared under a normal light/dark cycle and stained with anti-TRP antibodies (αTRP). (B) Sections were prepared from 7-d-old flies maintained constantly in the dark and stained with anti-TRP antibodies (αTRP). (C) Sections were prepared from flies reared under a light/dark cycle and stained with anti-INAD antibodies (αINAD). (D) Sections were obtained from 7-d-old dark-reared flies and stained with anti-INAD antibodies (αINAD). (E) Ultrastructure of single rhabdomeres from 1-d-old wild-type (wt), $trp^{\Delta 1272}$, (Δ1272), *norpA*, and *inaC* flies viewed by transmission electron microscopy. Bars: (A) 10 μm; (E) 0.5 μm.

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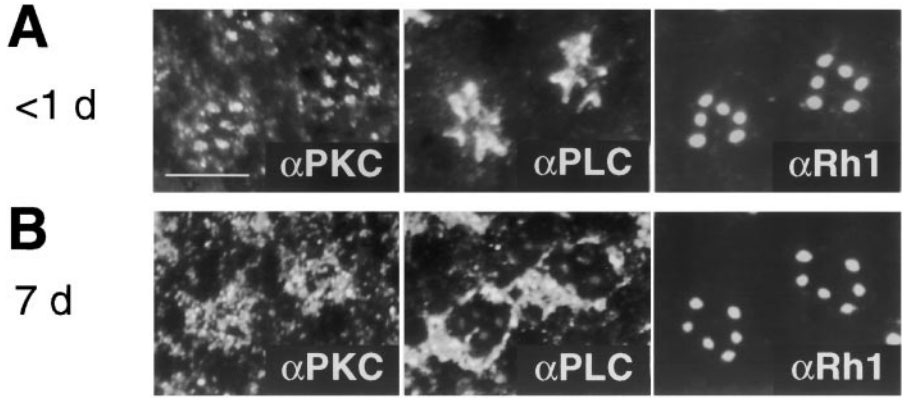

Figure 7. PLC and PKC were mislocalized in $trp^{\Delta 1272}$ flies. Tangential sections from 1- and 7-d-old $trp^{\Delta 1272}$ flies were stained with either anti-PLC (αPLC), anti-PKC (αPKC), or anti-Rh1 (αRh1) antibodies. Bar: 10 μm.

(Fig. 6, B and D). The proportion of TRP and INAD in the rhabdomeres of these latter flies was similar to that observed in young (<1 d after eclosion) $trp^{\Delta 1272}$ maintained under a light/dark cycle.

PLC and PKC were also mislocalized in old $trp^{\Delta 1272}$ flies; however, the time course and spatial distributions of PLC and PKC were distinct. In wild-type, PLC and PKC were localized specifically to the rhabdomeres (Fig. 3; Schneuwly et al., 1991; Smith et al., 1991). In young $trp^{\Delta 1272}$ photoreceptor cells, a high proportion of the PKC was also detected in the rhabdomeres, although a significant concentration of PKC was observed in the cell bodies (Fig. 7). By 7 d after eclosion, PKC was more uniformly distributed between the central matrix, the rhabdomeres, and cell bodies. The mislocalization of PLC in $trp^{\Delta 1272}$ was more rapid than for PKC, and was already pronounced in young $trp^{\Delta 1272}$. In older flies, the majority of the anti-PLC staining was found in the cell bodies. The mislocalization of TRP, INAD, PLC, and PKC in old $trp^{\Delta 1272}$ flies was specific to those proteins that depend on the signalplex for localization, as there was no perturbation in the rhabdomere-specific distribution of Rh1 (Fig. 7). Although INAD, PLC, and PKC were all mislocalized in $trp^{\Delta 1272}$, the time course was slightly slower than was the case in trp^{343}. In addition, INAD, PLC, and PKC were unstable in trp^{343} but not $trp^{\Delta 1272}$. These differences are most likely due to rhabdomeral degeneration in trp^{343}, as a rhabdomeral marker that does not depend on the signalplex for stability or localization, NINAC p174, decreases in an age-dependent manner in trp^{343} but not $trp^{\Delta 1272}$ (Fig. 5 B).

Normal Photoresponse in Young $trp^{\Delta 1272}$ Flies

The data presented above indicate that an important function of the TRP/INAD interaction is to retain both TRP and INAD in the rhabdomeres. If the primary function of the TRP/INAD association is to maintain these two proteins in the rhabdomeres, then young $trp^{\Delta 1272}$ flies would be expected to display a photoresponse similar to wild-type. According to this model, the photoresponse should be similar to wild-type, as the concentration of TRP and INAD in the rhabdomeres of young $trp^{\Delta 1272}$ is nearly normal. Alternatively, if the primary role of linking TRP directly to INAD is to facilitate rapid signaling, then the photoresponse should be disrupted in young $trp^{\Delta 1272}$ flies.

To address whether the INAD/TRP interaction has a primary role in the photoresponse, we performed ERG recordings. ERGs are extracellular recordings that measure the summed responses of all retinal cells to light. Exposure of wild-type flies to a bright orange-light stimulus results in a rapid corneal negative response because of influx of cations. Upon cessation of the stimulus, there is a rapid return of the maintained component to the baseline (Fig. 8 A). We found that ERGs recorded from young $trp^{\Delta 1272}$ flies were similar to wild-type, although the amplitude of the maintained component was slightly reduced (Fig. 8 A). In particular, and in contrast to young $InaD^{P215}$, there was no delay in termination of the photoresponse ($t_{1/3}$ = 89 ± 44 ms versus 1.55 ± 0.47 s; Fig. 8 A). In $InaD^{P215}$, exposure to a shorter light stimulus (0.7 versus 10 s) resulted in a less severe termination defect ($t_{1/3}$ = 0.53 ± 0.13 s versus 1.55 ± 0.47 s) (Fig. 8 B). Furthermore, in 7-d-old $trp^{\Delta 1272}$, there was no apparent defect in termination ($t_{1/3}$ = 138 ± 76 ms) of the photoresponse, although there was a pronounced decline in the amplitude of the maintained component (Fig. 8, A–C).

Discussion

Requirement for INAD for Retention Rather Than Targeting of TRP

The INAD signalplex has emerged as a paradigm for understanding the composition and function of a G protein–coupled macromolecular assembly. We have shown previously that INAD is required for the normal localization of TRP (Chevesich et al., 1997); however, these data did not discern whether the TRP/INAD interaction contributed to targeting or retention of TRP in the rhabdomeres. In this work, we found that TRP was initially localized to the rhabdomeres in young $InaD^{P215}$ and $trp^{\Delta 1272}$ flies, whereas in older flies, the spatial distribution of TRP was severely disrupted. These data suggested that INAD may be required for retention rather than targeting of TRP to the rhabdomeres. However, an alternative interpretation of these data is that those TRP molecules synthesized in young flies are targeted through an INAD-independent mechanism, whereas INAD is required for targeting of TRP synthesized in older flies. In support of the proposal that the INAD/TRP interaction was required for retention was the observation that TRP was long lived in vivo. TRP molecules synthesized before day 1.5 declined only ~25%

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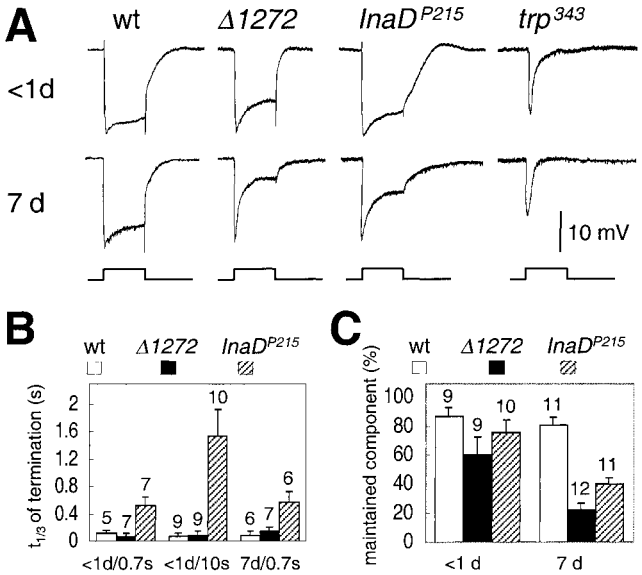

Figure 8. Photoresponse of $trp^{\Delta 1272}$ flies. (A) ERG recordings. White-eyed flies of the indicated genotypes and ages were stimulated with a 10-s pulse of orange light. wt, wild-type. (B) Histograms showing the time required for a 1/3 recovery after termination of the light stimuli ($t_{1/3}$). <1 d- and 7-d-old flies were stimulated for either 0.7 or 10 s as indicated. The $t_{1/3}$ in $InaD^{P215}$ flies was increased further by longer stimulation but not by age. $trp^{\Delta 1272}$ flies had no apparent termination defects. Error bars indicate the SEM, and the numbers of flies examined are listed above the error bars. wt, wild-type. (C) Histograms of the maintained components. The percentages represent the ratios of the amplitudes at the end of the 10-s stimulus divided by the peak amplitudes. wt, wild-type.

in concentration during the next 8 d. Thus, it appears that TRP is initially targeted to the rhabdomeres, and is subsequently mislocalized in the absence of a direct link to INAD.

TRP and INAD Form the Core Unit of the Signalplex

An intriguing question concerns the identification of proteins required for localization of INAD. The NINAC myosin III would appear to be an excellent candidate, as it binds INAD and is a putative molecular motor expressed in the photoreceptor cells. Nevertheless, NINAC is not required for localization of INAD (Wes et al., 1999). Other INAD-interacting proteins that function in phototransduction, such as rhodopsin, PLC, PKC, and TRPL, are also dispensable for rhabdomeral distribution of INAD. In contrast to these proteins, TRP was specifically required for proper localization of INAD. Moreover, in *trp* mutant flies and in transgenic flies, $trp^{\Delta 1272}$, in which the INAD binding site was deleted, the spatial distribution of INAD was disrupted in an age-dependent manner. These data, in combination with the findings that the half-life of INAD was ~5.5 d, suggested that the mislocalization of INAD in *trp* mutant flies was due to a defect in retention.

In addition to a requirement for the TRP/INAD interaction for localization of TRP and INAD, elimination of TRP or mutation of the INAD binding site in TRP led to an alteration in the spatial distribution of other proteins that require INAD for rhabdomere localization. These include PLC and PKC. Moreover, the mislocalization of PLC and PKC appeared to be more pronounced than that of INAD in young *trp* flies. One possibility is that these signaling proteins may also interact with TRP and depend on both TRP and INAD for localization. PKC appears to interact at least transiently with TRP, as TRP is a substrate for PKC. Thus, TRP and INAD appear to form the core unit that is required for localization of many of the signalplex components in the rhabdomeres.

The putative tetrameric structure of TRP may contribute to the stability of the TRP/INAD core unit, as each channel would have the potential to bind four INAD proteins. Although INAD was mislocalized in $trp^{\Delta 1272}$, there was no major impact on the localization of INAD in $InaD^{P215}$ (Chevesich et al., 1997), suggesting the existence of residual interaction between TRP and $INAD^{P215}$ in vivo. Consistent with this proposal, TRP was more unstable in $trp^{\Delta 1272}$ than in $InaD^{P215}$. The presumed tetrameric structure of TRP could enhance a weak interaction between $INAD^{P215}$ and TRP in vivo, which is not observed in pull-down assays using a monomeric TRP tail, as each channel would have the potential to bind four INAD proteins. The data presented here raise the possibility that other PDZ-containing scaffold proteins form similar core complexes in vivo with tetrameric ion channels. In support of this proposal are recent in vitro experiments indicating that there is a reciprocal requirement for localization of PSD-95 and the K^+ channel, Kv1.4 (Arnold and Clapham, 1999).

A separate question that awaits further investigation concerns the mechanism underlying targeting of the signalplex to the rhabdomeres. Evidence has been presented that another PDZ-containing scaffold protein, PSD-95, is trafficked to the postsynaptic compartment after assembling on vesicles (El-Husseini et al., 2000). Thus, it is plausible that the components of the signalplex may get trafficked to the rhabdomeres via vesicular transport and require the TRP/INAD core unit for retention.

TRP/INAD Association May Facilitate Retention of Proteins during Membrane Turnover

The finding that TRP and INAD were long lived was surprising considering that there is very active turnover of the rhabdomeric membrane (Blest, 1988). Such turnover results in shedding of rhabdomeral membrane into the central matrix and blebbing of membrane from the base of the microvilli into the cell bodies. The association between TRP and INAD may serve to prevent removal of these proteins into the central matrix and cell bodies during membrane turnover. Interestingly, the mutual requirement for the TRP/INAD interaction for retention in the rhabdomeres was less critical in $trp^{\Delta 1272}$ flies maintained in the dark. We suggest that a greater proportion of TRP and INAD was retained in the dark because of less turnover of the rhabdomeral membrane in the absence of light.

Direct TRP/INAD Interaction Is Not Required for the Photoresponse

In contrast to $InaD^{P215}$, the ERG response in young $trp^{\Delta 1272}$ was similar to wild-type. The only significant ERG

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phenotype in $trp^{\Delta 1272}$ flies was an age-dependent decrease in the amplitude of the maintained component. This defect was presumably due to mislocalization of TRP and INAD, as the amplitude of the maintained component gradually decreased in parallel with the mislocalization of TRP and INAD in older flies. Moreover, the termination of the photoresponse appeared normal even in old $trp^{\Delta 1272}$ flies. This latter result was surprising, as PKC is mislocalized in old $trp^{\Delta 1272}$ photoreceptor cells, and PKC is required for termination of the photoresponse (Smith et al., 1991; Hardie et al., 1993). However, the rhabdomeric concentration of two substrates for PKC, TRP (Huber et al., 1998) and INAD (Huber et al., 1996b), are also reduced in $trp^{\Delta 1272}$. Given that PKC, TRP, and INAD have been reported to be present in about equimolar concentrations (Huber et al., 1996a), we propose that the relative stoichiometry of PKC and its substrates is important for normal termination of the photoresponse. Thus, the delay in termination resulting from a reduction in PKC concentration may be suppressed by a concomitant decrease in the levels of INAD and TRP.

The defect in termination associated with $InaD^{P215}$ (Pak, 1979; Shieh and Zhu, 1996; Henderson et al., 2000) may not be due to perturbation of the TRP/INAD interaction, as the mutation in PDZ3 may also affect binding to other target proteins. The observation that the termination defect did not became more severe in old $InaD^{P215}$ flies suggested that the phenotype was not due to the disruption of the spatial distribution of TRP, as the mislocalization of TRP was more severe in older $InaD^{P215}$ flies.

To address the specific role of the TRP/INAD interaction, we mapped the INAD binding site and generated transgenic flies expressing a TRP derivative that did not associate with INAD. PDZ domains typically recognize COOH-terminal sequences consisting of an S/T-X-V/I motif or hydrophobic or aromatic residues (Kim et al., 1995; Kornau et al., 1995; Songyang et al., 1997; Daniels et al., 1998). As is the case with most PDZ target proteins, we found that the critical binding motif was at the COOH terminus. Specifically, we found that deletion of the last four amino acids (SGWL) completely disrupted TRP/INAD binding. Furthermore, $TRP^{\Delta 1272}$ obtained from transgenic fly head extracts failed to associate with INAD in pull-down assays. Mutation of an internal S-X-V motif (V1266D), which had previously been reported to abolish interaction between TRP and INAD in an overlay assay (Shieh and Zhu, 1996), had only minor effects. An additional deletion (amino acids 1257–1264), which eliminated the first residue of the S-X-V motif within the context of the short TRP tail (1252–1275), also retained binding with INAD in vitro (data not shown).

We conclude that the primary role of the direct interaction between TRP and INAD is not to facilitate rapid signaling. The apparently normal ERG in young $trp^{\Delta 1272}$ suggests that there is no defect in any aspect of the photoresponse. Rather, binding of TRP to INAD is critical for forming the core unit of the signalplex, which is necessary for retention of multiple signaling proteins in the rhabdomeres. This conclusion contrasts with the previous report, which concluded that INAD functions as a regulatory subunit of the TRP channel (Shieh and Zhu, 1996). Our analyses of $trp^{\Delta 1272}$ indicate that the delayed termination associated with $InaD^{P215}$ (Pak, 1979; Shieh and Zhu, 1996; Henderson et al., 2000) is not due to disruption of the interaction with TRP. Instead, it appears that the phenotype is due to disruption of the interaction of INAD with another signaling protein that is required for proper response termination. Thus, contrary to expectations, a direct association between TRP and INAD appears to be dispensable for rapid termination.

Multiple Functional Classes of INAD-binding Proteins

It appears that there are at least three classes of INAD binding proteins. The first class consists exclusively of TRP, as it is the only known INAD binding partner that is required for retention of INAD as well as of those INAD targets that depend on the signalplex for localization. However, there may be additional proteins that along with TRP and INAD comprise the core unit.

The second group includes two proteins, PLC and PKC, which rely on INAD for localization and stability. However, there is no reciprocal requirement for these proteins for retention of any other protein in the rhabdomeres. Mutation of the INAD binding sites in PLC (Shieh et al., 1997; van Huizen et al., 1998; Cook et al., 2000) and PKC (Adamski et al., 1998) have been reported to cause defects in the photoresponse. However, these effects may reflect mislocalization or instability of these INAD targets rather than a direct requirement for coupling to INAD.

The third class of INAD target proteins includes proteins such as rhodopsin, NINAC, and TRPL that are not dependent on INAD for localization in the rhabdomeres. We propose that the class I and II proteins, which depend on interaction with INAD for retention in the rhabdomeres, are constitutively bound to INAD, whereas the class III proteins may interact dynamically with INAD. As a consequence, only a subset of the class III proteins may bind to INAD at any given time. The observation that class III proteins do not depend on INAD for localization suggests that these INAD/target protein interactions have an alternative function, such as a direct role in the photoresponse. In support of this proposal, we have recently found that mutation of the INAD binding site in NINAC results in a pronounced delay in termination of the photoresponse (Wes et al., 1999). Thus, proteins that do not depend on INAD for localization may participate in the rapid activation and/or termination of the photoresponse.

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