Reversible Phosphorylation of the Signal Transduction Complex in Drosophila Photoreceptors

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In the Drosophila visual cascade, the transient receptor potential (TRP) calcium channel, phospholipase Cβ (no-receptor-potential A), and an eye-specific isoform of protein kinase C (eye-PKC) comprise a multimolecular signaling complex via their interaction with the scaffold protein INAD. Previously, we showed that the interaction between INAD and eye-PKC is a prerequisite for deactivation of a light response, suggesting eye-PKC phosphorylates proteins in the complex. To identify substrates of eye-PKC, we immunoprecipitated the complex from head lysates using anti-INAD antibodies and performed in vitro kinase assays. Wild-type immunocomplexes incubated with [32P]ATP revealed phosphorylation of TRP and INAD. In contrast, immunocomplexes from inaC mutants missing eye-PKC, displayed no phosphorylation of TRP or INAD. We also investigated protein phosphatases that may be involved in the dephosphorylation of proteins in the complex. Dephosphorylation of TRP and INAD was partially suppressed by the protein phosphatase inhibitors okadaic acid, microcystin, and protein phosphatase inhibitor-2. These phosphatase activities were enriched in the cytosol of wild-type heads, but drastically reduced in extracts prepared from glass mutants, which lack photoreceptors. Our findings indicate that INAD functions as RACK (receptor for activated PKC), allowing eye-PKC to phosphorylate INAD and TRP. Furthermore, dephosphorylation of INAD and TRP is catalyzed by PP1/PP2A-like enzymes preferentially expressed in photoreceptor cells.

Protein kinase C (PKC) modulates various biological processes by transferring phosphates to serine and threonine residues of substrates. Regulatory proteins such as RACK (receptor for activated PKC) and RICK (receptor for inactive PKC) have been shown to target PKC to specific subcellular compartments. This selective distribution of the kinase directs its catalytic activity toward restricted groups of substrates localizing proximal to the enzyme. The Drosophila visual cascade is a Gβγ-coupled phospholipase Cβ (PLCβ)-mediated pathway (see Ref. 2 for review). In this cascade, an eye-specific isoform of PKC (eye-PKC) negatively regulates visual signaling (3, 4). Eye-PKC is localized to the rhabdomere (4), a densely packed microvilli structure where visual transduction takes place, by tethering to the adaptor protein INAD (inactivation-no-after-potential D) (5).

INAD contains five distinct PDZ (Postsynaptic density 95; Discs large; and Zonula occludens 1) domains (5–8). PDZ domains are modular protein-protein interaction sequences consisting of 90–100 amino acids. Proteins containing PDZ domains have been implicated in clustering signaling molecules, including enzymes, receptors, and channels (9). PDZ domains mediate at least three types of protein-protein associations. The most common interaction is the binding of a PDZ domain to a tetrapeptide motif, X/S/T-X-φ-COOH (X, any amino acid; φ, hydrophobic residues), at the carboxyl terminus of target proteins (9, 10). The second type of interaction is exemplified by the association between α-syntrophin and neuronal nitric oxide synthase (nNOS). The crystal structure of the α-syntrophin/nNOS complex revealed that the PDZ domain of α-syntrophin interacts with an internal β-hairpin finger that is formed by two β-strands flanking the PDZ domain of nNOS (11). The third type of interaction by a PDZ domain is the binding to an internal S/T-X-V motif. The association between the third PDZ domain of INAD and transient receptor potential (TRP) (12) is one such example.

In Drosophila photoreceptors, INAD acts as a scaffold and interacts with eye-PKC (5, 8), TRP (12), and no-receptor-potential A (NORPA) (13–15). NORPA, a PLCβ (16), is essential for visual transduction; mutants lacking NORPA do not respond to light (17). TRP is a calcium channel responsible for the light-induced calcium influx leading to depolarization of photoreceptors (18–20). We previously reported that eye-PKC via its carboxyl terminus interacts with the second PDZ domain of INAD (8). To reveal the in vivo function of the INAD-PKC interaction, we generated transgenic flies expressing a modified eye-PKC containing a point mutation in the PDZ binding motif. Notably, these transgenic flies exhibit an abnormal deactivation phenotype identical to the eye-PKC null, indicating that a functional interaction between eye-PKC and INAD is essential for normal visual signaling (8). These findings indicate that the in vivo role of the INAD scaffold protein is to promote selective targeting of eye-PKC. The close proximity of the proteins in the INAD complex suggested to us that INAD, NORPA, and TRP could be potential substrates of eye-PKC in vivo. Indeed, all three proteins contain multiple consensus phosphorylation sites for PKC (16, 18, 19, 21). To investigate how eye-PKC regulates visual transduction, we investigated phosphorylation of the proteins in the INAD complex isolated from wild-type flies and several mutants having defects in visual transduction. Results indicate that both INAD and TRP are phosphorylated by eye-PKC in a

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complex-dependent manner. We also examined phosphatases involved in the dephosphorylation of TRP and INAD. Inhibitor studies indicate that dephosphorylation of INAD and TRP may be regulated by serine/threonine protein phosphatases 1 and 2A (PP1 and PP2A)-like enzymes expressed in wild-type but not in glass heads.

EXPERIMENTAL PROCEDURES

Materials—Triton X-100, Tween 20, and protein A-Sepharose were obtained from Sigma. Dithiothreitol (DTT) and protease inhibitors (leupeptin, aprotinin, pepstatin A, and phenylmethylsulfonyl fluoride) were purchased from United States Biochemicals (Cleveland, OH). Benzamidine and benzamidine were from Fluka (Milwaukee, WI). Okadaic acid was from LC laboratories (Woburn, MA). Microcystin LR, protein phosphatase inhibitor-2 (PPI-2), and cypermethrin were from Calbiochem (San Diego, CA). Alkaline phosphatase-conjugated goat anti-rabbit IgG and other secondary antibodies were from Jackson Immunoresearch (San Diego, CA). Alkaline phosphatase-conjugated goat anti-rabbit IgG and other secondary antibodies were from Jackson Immunoresearch Laboratory (West Grove, PA). Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were purchased from Research Organics (Cleveland, OH). [γ-32P]ATP (6000 Ci/mmol) was from New Life Science Products. Polyclonal antibodies against INAD and TRP were prepared as described previously (12).

Preparation of Fly Extracts—Fly heads were either isolated in mass by the use of screens or dissected manually under CO2 anesthesia. Approximately 100 wild-type heads were homogenized with 0.3 ml of extraction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and a mixture of protease inhibitors containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and pepstatin A, 0.5 μg/ml aprotinin, 0.01 mM benzamide, and 1 mM benzamidine) with or without detergents (1% Triton X-100, 0.1% Tween 20, 0.1% Nonidet P-40) as indicated. Head homogenates were incubated at 4 °C with constant agitation for 1 h for extraction of the INAD complex. The mixture was then centrifuged for 10 min (12,000 × g), and the supernatant was used for immunoprecipitation.

Immunoprecipitation and In Vitro Kinase Assay—Immunoprecipitations were performed as described previously (12). Immunocomplexes were incubated with 50 μl of phosphorylation buffer (50 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 5 mM 2-mercaptoethanol, 0.1 mM DTT, 0.4 mM EGTA, 0.7 mM CaCl2, and 3 μCi of carrier-free [γ-32P]ATP at 30 °C for various lengths of time. Phosphorylation reactions were terminated by addition of 2× SDS-PAGE loading buffer. Protein phosphorylation was monitored by autoradiography or PhosphorImager analysis following SDS-PAGE and Western blotting. For each experiment, at least three independent assays were carried out and one representative result was shown in the figures.

Western Blotting and Protein Determination—Western analysis was performed as described previously (12). The level of proteins in the blots was quantitated by Image analysis software (ImageQuanNT) following scanning. Protein concentrations of extracts were routinely determined by the Bio-Rad protein assay using bovine serum albumin as a standard.

Fly Stocks—Fly stocks were maintained at 25 °C in a 12-h dark/12-h light cycle.

RESULTS

Time Course of Complex-specific Phosphorylation of TRP and INAD—In Drosophila photoreceptors, the association between INAD and eye-PKC is essential for the in vivo kinase activity, which regulates deactivation of visual signaling (8). INAD forms a signaling complex by tethering eye-PKC, TRP, and NORPA. As a result of the close proximity of NORPA, TRP, and INAD to eye-PKC, these proteins could be eye-PKC substrates. Therefore, we investigated protein phosphorylation of the INAD-associated proteins isolated by immunoprecipitation. We found that TRP (12), NORPA (13), and eye-PKC (data not shown) were immunopurified from wild-type fly head extracts using anti-INAD antibodies. However, we did not detect rhodopsin, Gαq, or TRPL in the complex (data not shown) as observed by others (14). Analysis of the kinase activity in the immunocomplexes revealed phosphorylation of both INAD and TRP (Fig. 1A). Phosphorylation of NORPA was not readily detected under the same conditions, perhaps due to the sensitivity of the assay. Maximum phosphorylation of TRP and INAD occurred following 30 min of incubation at 30 °C (Fig. 1B).

The Complex-specific Phosphorylation Is Dependent on Eye-PKC—To investigate how complex-specific phosphorylation may be regulated, immunoprecipitation and kinase assays were carried out on several mutant extracts lacking specific components of the visual cascade. Importantly, the observed phosphorylation of INAD and TRP was dependent on the presence of eye-PKC; INAD complexes isolated from inac flies missing eye-PKC were incapable of promoting phosphorylation of these two proteins (Fig. 2A, lane 4). In contrast, phosphoryl-
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Dephosphorylation of INAD and TRP Is Independent of Divalent Cations—The four major enzyme classes responsible for phosphoserine and phosphothreonine dephosphorylation are PP1, PP2A, PP2B, and PP2C. These enzymes can be distinguished from one another by their divalent cation dependence and their sensitivities to inhibitors (22). PP2B and PP2C exhibit a strict requirement for Ca$^{2+}$ and Mg$^{2+}$ respectively, whereas PP1 and PP2A do not require divalent cations for enzymatic activity. To gain insight into which class of phosphatase is involved in dephosphorylation of INAD and TRP, we first determined the requirement of divalent cations for dephosphorylation of INAD and TRP. As shown in Fig. 4B, we did not observe any enhancement of dephosphorylation upon addition of Mg$^{2+}$, Ca$^{2+}$, and Mn$^{2+}$ ($20 \text{ mM}$) to the detergent-soluble extracts of wild-type fly heads. These results suggest that members of the PP1 and PP2A families are involved in dephosphorylation of TRP and INAD, because the enzymatic activities of these protein serine/threonine phosphatases are independent of divalent cations (23).

Dephosphorylation of INAD and TRP: Inhibitor Studies—We further investigated which class of phosphatases is involved in dephosphorylation of INAD and TRP by the use of four phosphatase inhibitors, including okadaic acid, microcystin, inhibitor-2 (PPI-2), and cypermethrin. Cypermethrin (40 pM), a potent inhibitor of PP2B (24), failed to prevent dephosphorylation of INAD and TRP (data not shown). Both okadaic acid and microcystin, on the other hand, inhibit PP2A at low concentrations ($IC_{50} = 0.1 \text{ nM}$ for okadaic acid, $IC_{50} = 0.04 \text{ nM}$ for microcystin), and PP1 at higher concentrations ($IC_{50} = 10 \text{ nM}$ for okadaic acid, $IC_{50} = 1.7 \text{ nM}$ for microcystin) (25, 26). As shown in Fig. 5A, 0.1 nM okadaic acid partially suppressed the dephosphorylation of TRP and INAD in Triton X-100 extracts, indicating that these proteins are potential targets for PP2A-like enzymes. At 10 nM, okadaic acid displayed a slightly greater inhibitory effect, suggesting the phosphatases have
Dephosphorylation reactions were carried out for 8 min at 30 °C in the presence and absence of various divalent cations as indicated below, and the remaining 32P per unit protein are shown as a percentage of the negative control.

As shown in Fig. 6, both cytosolic and membrane extracts from glass flies that lack photoreceptors (27). As shown in Fig. 4C, moreover, results obtained from studies using microcystin (Fig. 5B) also suggested a role for PP2A/PP1-like enzymes in the dephosphorylation.

Both TRP and INAD Phosphatases Are Enriched in Photoreceptors—To investigate the subcellular distribution of the INAD/TRP phosphatases, we analyzed both cytosolic and membrane extracts of wild-type fly heads. These two extracts were prepared by sequential solubilization of fly head proteins first with a buffer containing no detergents ("cytosolic extracts"); followed by a buffer containing 1% Triton X-100 ("membrane extracts") (see "Experimental Procedures"). Taken together, our results strongly support the notion that PP1/PP2A-like enzymes regulate the dephosphorylation of TRP and INAD.

Dephosphorylation of TRP and INAD could be mediated by photoreceptor-specific phosphatases or by ubiquitously expressed enzymes present in many cell types. To distinguish between these two possibilities, we examined phosphatase activities from glass flies that lack photoreceptors (27). As shown in Fig. 6, both cytosolic and membrane extracts from glass mutant fly heads showed little phosphatase activity as compared with cytosolic extracts from wild-type heads. These findings indicate that dephosphorylation of INAD and TRP are regulated by photoreceptor-specific phosphatases.

**DISCUSSION**

The reversible phosphorylation of proteins regulates a vast array of biological processes. Protein phosphorylation mediated by members of the protein kinase C family is involved in both cell growth and receptor-mediated signal transduction (28). In Drosophila photoreceptor cells, eye-PKC serves to modulate visual transduction; inaC mutants that lack the kinase display abnormal deactivation and light adaptation (3). Moreover, photoreceptors in inaC mutants undergo light-dependent degeneration (4). Insights into the identity of eye-PKC substrates, as well as the protein phosphatases that dephosphorylate these substrates, should lead to a better understanding of regulation of the visual cascade.

Recent evidence indicates that eye-PKC, via its association with INAD, is tethered to a signaling complex consisting of INAD, NORPA, and TRP (5, 12–15, 29, 30). We previously showed that a lack of interaction between eye-PKC and INAD leads to a complete loss of the in vivo activity of eye-PKC (8). These findings suggest that proper subcellular localization of eye-PKC, via its interaction with INAD, targets the kinase to its substrates. Although INAD and TRP have been shown to be phosphorylated in vivo and in vitro in the blowfly Calliphora (29, 31), the identity of the kinase was not known. In this report, we combined an in vitro kinase assay with immunoprecipitation to identify substrates of eye-PKC. We show that both INAD and TRP become phosphorylated and that phosphorylation of these two proteins is dependent on the presence of eye-PKC in the complex. These results demonstrate that both INAD and TRP are substrates of eye-PKC in vitro and probably in vivo. NORPA (PLCβ), another member of the signaling complex, is responsible for catalyzing the hydrolysis of phospholipids to generate diacylglycerol, which leads to activation of eye-PKC. Although NORPA is an ideal candidate for eye-PKC-mediated feedback regulation, it was not phosphorylated under the experimental conditions described in this report. It remains a possibility that a limited number of phosphorylation sites on NORPA eluded detection by our assays or that there is a different kinase, not associated with INAD, responsible for phosphorylation of NORPA.

Phosphorylation of the scaffold protein INAD may have functional implications in modulating protein-protein interactions. For example, the interaction between PSD95 and the inward rectifying K+ channel Kir2.3 appears to be regulated by phosphorylation (32). Similarly, phosphorylation of INAD in the PDZ domain anchoring eye-PKC may promote dissociation of the kinase from the complex. There are seven putative protein kinase C phosphorylation sites in INAD, including two in the
second PDZ domain (21) which tethers eye-PKC. On the other hand, phosphorylation of TRP, the major ion carrier underlying light-induced depolarization (20), may affect the kinetics of the calcium channel. For example, a lack of eye-PKC-mediated phosphorylation of TRP may result in abnormal deactivation similar to that observed in $\text{InaD}^{215}$. At present, the mechanism by which TRP operates remains under intense investigation. Knowledge of the eye-PKC phosphorylation sites should help elucidate the gating mechanisms of TRP.

Efficient and timely phosphorylation of TRP and INAD is dependent upon the close proximity of eye-PKC to these substrates. It is equally important that dephosphorylation is carried out in a timely manner to speed up recovery from a light response. The co-localization of protein phosphatases and protein kinases by scaffold proteins (33, 34), or the tight association of kinases and phosphatases (35), may facilitate fast dephosphorylation, thus reversing the action of protein kinases. However, we find that protein phosphatases do not appear to be tightly associated with the INAD signaling complexes in $\text{Drosophila}$ photoreceptors. Indeed, phosphatases that are involved in dephosphorylation of INAD and TRP are present in the cytosol. Moreover, only cytosolic extracts prepared from wild-type but not from $\text{glass}$ heads contain the desired phosphatase activities.

It is noteworthy that $\text{in vitro}$ phosphorylation and dephosphorylation reactions proceed at a much slower rate than those likely to occur $\text{in vivo}$ for regulating visual signaling. The reduced rate of these $\text{in vitro}$ assays may result from partial inactivation of eye-PKC and protein phosphatases in the extract following homogenization and dilution. Moreover, differences in the kinetics of phosphorylation may be due to the fact that eye-PKC in isolated complexes are not subject to negative regulation by phosphatases in our $\text{in vitro}$ phosphorylation assays, because of the absence of phosphatases in the complex.

Based on their requirement for divalent cations and sensitivity to inhibitors (23), protein serine/threonine phosphatases are subdivided into four major classes: PP1, PP2A, PP2B, and PP2C. PP1 and PP2A are sensitive to okadaic acid and microcystin and do not require divalent cations for enzymatic activity (23). These protein phosphatases consist of multiple subunits; both PP1 and PP2A catalytic subunits form complexes...
with tissue-specific targeting subunits that are important for substrate recognition (36). To date, the catalytic subunits of PP1 and PP2A identified in Drosophila appear to be expressed ubiquitously (37). However, dephosphorylation of INAD and TRP was involved with photoreceptor-specific PP1/PP2A-like phosphatases absent in glass extracts. The glass gene encodes a zinc finger transcription factor that switches on gene expression of rhodopsin and proteins involved in the visual cascade. Knowledge of photoreceptor-specific protein phosphatases may lead to a better understanding of the regulation of visual signal transduction by protein phosphatases.

In summary, we show that both INAD and TRP are phosphorylated in a complex-specific manner. Furthermore, this phosphorylation is dependent on the presence of eye-PKC in photoreceptor cells. Future studies on the molecular characterization of these phosphatases, and the identification of phosphorylation sites in INAD and TRP, will lead to a better understanding of the regulation of visual signal transduction by protein phosphatases.

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