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Light-Induced Recruitment of INAD-Signaling Complexes to Detergent-Resistant Lipid Rafts in *Drosophila* Photoreceptors

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Abstract

Here, we reveal a novel feature of the dynamic organization of signaling components in *Drosophila* photoreceptors. We show that the multi-PDZ protein INAD and its target proteins undergo light-induced recruitment to detergent-resistant membrane (DRM) rafts. Reduction of ergosterol, considered to be a key component of lipid rafts in *Drosophila*, resulted in a loss of INAD-signaling complexes associated with DRM fractions. Genetic analysis demonstrated that translocation of INAD-signaling complexes to DRM rafts requires activation of the entire phototransduction cascade, while constitutive activation of the light-activated channels resulted in recruitment of complexes to DRM rafts in the dark. Mutations affecting INAD and TRP showed that PDZ4 and PDZ5 domains of INAD, as well as the INAD-TRP interaction, are required for translocation of components to DRM rafts. Finally, selective recruitment of phosphorylated, and therefore activatable, eye-PKC to DRM rafts suggests that DRM domains are likely to function in signaling, rather than trafficking.

Introduction

Drosophila photoreceptors have served as an excellent model system for studying both G-protein coupled signaling mechanisms as well as the intracellular organization of signaling components (Hardie and Raghu, 2001; Ranganathan et al., 1995a; Tsunoda and Zuker, 1999). Signaling occurs within a specialized subcellular compartment, the rhabdomere, which consists of ~60,000 tightly packed microvilli that house most phototransduction components. Within the rhabdomere, the scaffold protein INAD (inactivation-no-afterpotential-D) brings components into close proximity with one another, promoting a high speed of signaling (Huber, 2001; Ranganathan and Ross, 1997; Tsunoda et al., 1998; Tsunoda and Zuker, 1999). Phototransduction in *Drosophila* is triggered by the photo-isomerization of the light receptor, rhodopsin, to its active form, meta-rhodopsin. Meta-rhodopsin activates a G_qα protein that, in turn, stimulates the effector protein, phospholipase Cβ (PLC). Activation of PLC leads to the eventual opening of two cationic channels, transient-receptor-potential (TRP) and TRP-like (TRPL). The organizer INAD contains five PDZ (PSD-95, Dlg, ZO-1) domains, which interact with PLC, TRP, and an eye-specific protein kinase-C (eye-PKC). PLC has been shown to bind PDZ1 and PDZ5 (Shieh et al., 1997; Tsunoda et al., 1997; van Huizen et al., 1998), TRP has been shown to bind PDZ3 (Shieh and Zhu, 1996; Tsunoda et al., 1997), and eye-PKC has been

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shown to bind PDZ2 and PDZ4 (Adamski et al., 1998; Tsunoda et al., 1997). In an *inaD* null mutant, PLC, TRP, and eye-PKC are completely mislocalized, and consequently, light responsiveness is severely impaired (Tsunoda et al., 1997), demonstrating the importance of assembling signaling complexes and localizing them to the rhabdomere.

Studies in a variety of cell types and organisms have shown that lipid rafts function as microdomains or platforms involved in signal transduction, protein sorting and trafficking (Golub et al., 2004; Lai, 2003; Simons and Toomre, 2000). Lipid rafts, which are found in the outer leaflet of the Golgi and plasma membranes, have been shown to be enriched in cholesterol, sphingolipids, and GPI-linked proteins (Harder and Simons, 1997; Lai, 2003; Simons and Toomre, 2000). It is the liquid ordered packing of cholesterol, or ergosterol in the cases of *Drosophila* and yeast, with sphingolipids that is thought to make lipid rafts resistant to cold detergent solubilization (Brown and London, 1997, 1998; Brown and Rose, 1992; Hooper, 1999; Simons and Ikonen, 1997; Simons and van Meer, 1988). In vertebrate photoreceptors, detergent-resistant membrane (DRM) rafts have been found to constitutively house some phototransduction components, while recruiting others, including transducin, RGS9-1-Gβ5L, and arrestin, upon illumination (Nair et al., 2002). Since DRM rafts have recently been isolated from *Drosophila* and implicated in signaling (Eroglu et al., 2003; Hoehne et al., 2005; Rietveld et al., 1999; Zhai et al., 2004), we set out to explore whether signaling components in *Drosophila* photoreceptors might be associated with lipid rafts.

Recent studies demonstrating that light regulates the availability of some phototransduction components by inducing their subcellular translocation have brought attention to the dynamic nature of signaling components in photoreceptors. In this study, we show that light induces the translocation of the scaffold protein INAD and its target proteins, PLC, TRP, and eye-PKC, to DRM rafts in the rhabdomeres of photoreceptors. In contrast, the major rhodopsin Rh1 and G_qα remain in non-raft membrane domains of the rhabdomere, regardless of light condition. We show that recruitment to DRM rafts is dependent on ergosterol, the PDZ4 and PDZ5 domains of INAD, as well as the INAD-TRP interaction. Genetic analysis also demonstrates that activation of the entire phototransduction cascade is required for the translocation of INAD-signaling complexes to DRM rafts. Consistent with these results, *rdgA* mutants, which exhibit constitutive activation of TRP and TRPL channels, display constitutive recruitment of INAD-signaling complexes to DRM rafts in the dark. Finally, selective recruitment of the phosphorylated, and therefore mature and activatable, form of eye-PKC to DRM rafts suggests that DRM domains are likely to function in a signaling, rather than trafficking, capacity.

Results

Light-Dependent Recruitment of INAD-Signaling Complexes to DRM Rafts

Since lipid rafts have been shown to function as platforms involved in signal transduction and protein trafficking, we examined whether signaling proteins in *Drosophila* photoreceptors are present in lipid rafts. In mammals, the ordered packing of cholesterol and sphingolipids is thought to make lipid rafts resistant to cold detergent (Triton-X 100) treatment (Brown and Rose, 1992; Sankaram and Thompson, 1990; Schroeder et al., 1994). In *Drosophila*, ergosterol, rather than cholesterol, is the major sterol present (Eroglu et al., 2003; Rietveld et al., 1999), and previous studies have predicted that ergosterol functions similarly to cholesterol in the formation of lipid rafts (Eisenkolb et al., 2002; Rietveld et al., 1999; Xu et al., 2001). Indeed, DRMs have been isolated from *Drosophila* in several recent studies (Eroglu et al., 2003; Hoehne et al., 2005; Rietveld et al., 1999; Zhai et al., 2004). We set out to investigate whether signaling components in *Drosophila* photoreceptors associate with DRMs either statically in the dark, or in a manner regulated by light-exposure. First, we isolated membranes from dark-raised wild-type flies, solubilized membranes with 1% Triton-X 100 on ice, and subjected samples to an Optiprep density floatation gradient (see Experimental Methods). Six fractions

were taken from top to bottom of the gradient, numbered #1 to 6, respectively. Non-raft proteins are expected to be solubilized by the cold detergent treatment and therefore remain at the bottom of this gradient in higher density fractions. In contrast, lipid raft membranes, which are resistant to cold detergent solubilization, are expected to float to light density fractions. The six fractions were analyzed by immunoblot analysis to determine if phototransduction components were associated with light density DRM fractions.

We found that the major rhodopsin (Rh1) and $G_q\alpha$ proteins were consistently present in fractions #5–6 (Fig. 1), suggesting that Rh1 and $G_q\alpha$ were solubilized by cold detergent treatment and therefore not associated with lipid rafts. The solubilization of such an abundant transmembrane protein ($\sim 10^6$ Rh1/photoreceptor) suggests that other non-raft proteins present were also likely to be solubilized in these conditions. We next examined whether INAD-signaling complexes were associated with light density DRMs. We found that the scaffold protein INAD and its target proteins, PLC, TRP, and eye-PKC were primarily present in the heavier density fractions #4–5, with a small quantity of eye-PKC and INAD found in fractions #1–2 (Fig. 1). These results suggest that in the dark, Rh1, $G_q\alpha$, and the INAD-signaling complex are primarily associated with non-raft membrane domains.

To determine if signaling components in *Drosophila* photoreceptors are recruited to DRM with light-exposure, we subjected dark-raised flies to two hours of white light (see Experimental Methods). Membranes were isolated, subjected to cold detergent treatment, and analyzed as described above. Rh1 and $G_q\alpha$ were again solubilized by detergent treatment and showed no recruitment to light density raft fractions. TRPL channels, which are not considered a part of the core INAD-signaling complex, were also solubilized in both dark and light conditions (data not shown). In contrast, INAD, PLC, TRP and eye-PKC all showed significant recruitment to lighter density fractions #1–2 when flies were light-exposed (Fig. 1). Since live flies were either dark-raised or light-exposed, and membranes were isolated and analyzed similarly, differences are likely to represent *in vivo* effects of light stimulation, suggesting that INAD, PLC, TRP, and eye-PKC, are specifically recruited to DRMs with light *in vivo*.

We next set out to confirm that these signaling components in light density fractions #1–2 were indeed associated with lipid rafts. Unfortunately, the well-established lipid raft markers used in mammalian cells were either not available or not applicable for adult *Drosophila*. Thus, we used another criterion to distinguish lipid raft domains: disassociation of DRM rafts after detergent treatment at 37°C (Brown and London, 1997; Hooper, 1999; Simons and Toomre, 2000). We tested light-exposed flies for recruitment of INAD-signaling complexes to light density fractions after detergent treatment at 37°C. We found that INAD, PLC, TRP, and eye-PKC all failed to associate with light density fractions #1–2 when treated with Triton-X 100 treatment at 37°C (Fig. 1). These results further suggest that INAD-signaling complex components are recruited to lipid rafts.

Disruption of INAD-Signaling Complex Recruitment to DRM Rafts when Dietary Ergosterol is Limited

To further confirm that phototransduction components in fractions #1–2 were indeed associated with lipid rafts, we sought to disrupt lipid rafts and then test for the presence or absence of components in these fractions. Although methyl- β -cyclodextrin (MCD) is commonly used to disrupt rafts in mammalian cells by binding cholesterol (Brown and London, 2000; Simons and Toomre, 2000), MCD is unable to form complexes with ergosterol, making it unlikely to disrupt rafts in *Drosophila* membranes. Consistent with this prediction, our attempts to use MCD to disrupt rafts in *Drosophila* membranes have been met with limited success (data not shown).

Thus, we set out to disrupt lipid rafts using an alternative approach –by depleting ergosterol, the major sterol in *Drosophila* and the sterol predicted to function in the formation of lipid rafts. Since *Drosophila* obtain sterols exclusively from their diet, we attempted to disrupt ergosterol-based lipid rafts in the membranes of *Drosophila* by limiting the ergosterol intake of flies. Since yeast contain ergosterol as their major membrane sterol, laboratory flies obtain ergosterol primarily from the yeast included as a major component of most laboratory fly food (see Experimental Methods). Yeast, in contrast to *Drosophila*, depend entirely on endogenous ergosterol biosynthesis (Keesler et al., 1992; Trocha and Sprinson, 1976). To limit the ergosterol intake of flies, we prepared a more minimal fly food, using mutant yeast strains deficient for enzymes in the ergosterol biosynthetic pathway (see Experimental Methods). We used mutant yeast strains *erg2Δ*, *erg6Δ*, and *erg2Δerg6Δ* (generated and kindly provided to us by Dr. Howard Riezman; (Munn et al., 1999). *erg2Δ* and *erg6Δ* mutants lack C-8 sterol isomerase and C-24 sterol methyltransferase activity, respectively, which individually and combinatorially prevent the biosynthesis of ergosterol (Munn et al., 1999). Although these mutants do accumulate other sterols, these mutations have been shown to affect lipid raft maintenance (Eisenkolb et al., 2002). Wild-type flies were then fed a special food containing mutant yeast *erg2Δ*, *erg6Δ*, or *erg2Δerg6Δ* for one month (*ergΔ*-fed flies; see Experimental Methods). To determine whether these *ergΔ*-fed flies did indeed exhibit decreased levels of ergosterol, we quantitated and compared the ergosterol content of whole fly extracts from flies grown on our standard laboratory fly food containing wild-type yeast with *ergΔ*-fed flies. Sterols were extracted from fly homogenates and subjected to ultraviolet spectrophotometric analysis. Ergosterol was identified based on its unique absorbance profile from 250 to 300 nm, similar to previous studies (Arthington-Skaggs et al., 1999); figure 2A shows the characteristic four-peaked profile of ergosterol. UV spectrophotometric profiles for flies grown on regular laboratory fly food exhibited the greatest absorbance at all wavelengths, while decreased absorbance was observed for all *ergΔ*-fed flies (Fig. 2A). To quantitate ergosterol content, absorbance readings were taken at 281.5 nm, which are predicted to correspond to ergosterol and the late sterol intermediate 24(28)DHE (Arthington-Skaggs et al., 1999). To determine whether there were significant levels of DHE present that would interfere with our quantitation of ergosterol, we also took absorbance readings at 230 nm, which should only reflect DHE levels. No clear absorbance peak, however, was detected at 230 nm (data not shown), consistent with previous reports that ergosterol is the predominant sterol present in *Drosophila* membranes (Eroglu et al., 2003; Hoehne et al., 2005; Rietveld et al., 1999; Zhai et al., 2004). To estimate the differences in ergosterol content, relative average absorbances at 281.5 were calculated for each sample, as compared to wild-type. We found that, indeed, *ergΔ*-fed flies contained $53.34 \pm 10.6\%$, $38.35 \pm 2.4\%$, and $59.42 \pm 4.2\%$ (for *erg2Δ*, *erg6Δ*, and *erg2Δerg6Δ*, respectively) the amount of ergosterol found in flies fed on regular laboratory fly food (Fig. 2B). Thus, feeding flies food made from *ergΔ* mutant yeast strains for one month was an effective method for significantly reducing the ergosterol content of flies.

To examine whether the recruitment of INAD-signaling complexes to lipid rafts was disrupted in *ergΔ*-fed flies, membranes from light-exposed *ergΔ*-fed flies were isolated, subjected to detergent treatment at 4°C, and analyzed in density floatation assays. In contrast to flies fed wild-type yeast food, *ergΔ*-fed flies all showed severely decreased or completely abolished translocation of INAD, PLC, TRP, and eye-PKC to light density raft fractions (Fig. 2C). Small differences in residual quantities of INAD, PLC, TRP, and eye-PKC associated with light density fractions in flies fed *erg2Δ*, *erg6Δ*, or *erg2Δ erg6Δ* yeast may be due to incomplete replacement of ergosterol incorporated into cells during embryonic and larval stages, or to differences in the kinds of sterols accumulated in each of these mutant yeast strains. For example, the most abundant sterols present are: fecosterol (33.2%) and ergosta-8-enol (35.4%) in *erg2Δ*, zymosterol (39.4%) and cholesta-5,7,24-trienol (32.2%) in *erg6Δ*, and zymosterol (85.6%) in *erg2Δerg6Δ* mutants (Munn et al., 1999). It is likely that these different sterols support raft formation to different degrees. The near complete elimination of INAD, PLC, TRP,

and eye-PKC from light density raft fractions after cold detergent treatment, however, suggests that the light-induced recruitment of INAD-signaling complexes to DRM domains is dependent on ergosterol. These results are consistent with our proposal that INAD-signaling complexes are indeed recruited to ergosterol-based lipid raft domains.

Subcellular Localization of Sterol-Rich Domains in Photoreceptors

To examine where lipid raft domains might be localized in photoreceptors, we used the fluorescent properties of filipin, which complexes with sterols containing a free 3 β -hydroxyl group, such as ergosterol and cholesterol, in membranes (Norman et al., 1972). Filipin has been used in previous studies to identify subcellular domains rich in lipid rafts (Bagnat and Simons, 2002; Simons and Toomre, 2000; Takeda and Chang, 2005; Wachtler et al., 2003). We examined filipin treated retinal sections from dark-raised wild-type flies to determine where sterol-rich domains are localized in photoreceptors. We found that sterol-rich domains labeled by filipin were primarily localized in the signaling compartment of the photoreceptor cell –the rhabdomere (Fig. 3). Filipin staining of tissue sections from dark-raised and light-exposed flies were similar (Fig. 3), suggesting that lipid raft domains do not undergo any major changes in localization with light-exposure. Since INAD is also localized to the rhabdomeres of both dark-raised and light-exposed photoreceptors (Fig. 3), INAD-signaling complexes are likely to be recruited to lipid rafts within the rhabdomeric membrane.

INAD is Required for the Recruitment of PLC, TRP, and eye-PKC to Lipid Rafts

Since the scaffold protein INAD is required for assembly and rhabdomeric localization of PLC, TRP, and eye-PKC, we investigated whether INAD might also be required for the light-induced recruitment of PLC, TRP, and eye-PKC to lipid rafts. Membranes isolated from dark-raised and light-exposed *inaD*¹ null mutants were subjected to detergent treatment and floatation through a density gradient. Since levels of PLC, TRP, and eye-PKC are progressively reduced with age in *inaD*¹ null mutants, we used flies that were less than 24 hours old, and a larger quantity (1.5X) of membranes, to minimize the loss of these target proteins (Tsunoda et al., 1997). PLC, TRP, and eye-PKC from dark-raised *inaD*¹ null mutants showed a distribution in non-raft fractions similar to dark-raised wild-type flies (data not shown). We next examined preparations from light-exposed *inaD*¹ null mutants and found that PLC, TRP, and eye-PKC all remained primarily associated with non-raft fractions (Fig. 4). These results show that INAD is essential for the light-induced recruitment of target proteins to lipid rafts, suggesting that complex assembly and/or rhabdomeric localization is important for recruitment to DRM rafts.

To begin to examine which domains of INAD are required for association with DRM rafts, we generated a transgenic line, *inaD*^{PDZ123-1D4}, which encodes the N-terminal PDZ1 to PDZ3 domains, but lacks PDZ4 and PDZ5 domains, fused to a six-residue epitope tag, 1D4, taken from the C-terminus of bovine rhodopsin. We found that the INAD^{PDZ123-1D4} protein displayed normal localization to the rhabdomeres of photoreceptors (Fig. 4B), suggesting that the PDZ4 and PDZ5 domains of INAD are not required for rhabdomeric localization. We then examined whether INAD^{PDZ123-1D4}, PLC, TRP, and eye-PKC were able to undergo light-induced translocation to DRM rafts. Membranes from light-exposed *inaD*^{PDZ123-1D4} mutant flies were isolated, subjected to cold detergent treatment, and analyzed in density floatation assays. We found that INAD^{PDZ123-1D4}, PLC, TRP, and eye-PKC all failed to associate with DRM fractions (Fig. 4). These results show that even though the INAD^{PDZ123-1D4} protein is localized to the rhabdomere, the PDZ4 and PDZ5 domains of INAD are required for the association of INAD with DRM rafts. Furthermore, our results suggest that the inability of the scaffold protein to translocate to DRM rafts prevents the recruitment of its target proteins to DRM rafts as well.

To examine the possibility that rhabdomeric localization is essential for components to be recruited to DRM rafts, we examined two transgenic lines, *inaD^{PDZ3}* and *trp^{C34}*, which were previously designed to disrupt the interaction between INAD and TRP, resulting in the mislocalization of INAD-signaling complexes (Tsunoda et al., 2001). The *inaD^{PDZ3}* transgenic line expresses an INAD^{PDZ3} protein with three point mutations in its PDZ3 domain (Ile375Ala, Ile377Ala, Val379Ala), which are predicted to disrupt interaction with TRP channels (Tsunoda et al., 2001). Consistent with the previous finding that TRP is essential for the rhabdomeric localization of INAD-signaling complexes, INAD^{PDZ3}-signaling complexes have also been shown to be mislocalized (Tsunoda et al., 2001). The *trp^{C34}* transgenic line expresses a truncated TRP protein that lacks the INAD binding site (Shieh and Zhu, 1996), also resulting in the mislocalization of INAD-signaling complexes (Tsunoda et al., 2001). We found that although PLC and eye-PKC are expected to still be assembled in complexes with INAD in these transgenic lines, light-exposure was unable to induce the translocation of PLC, eye-PKC, as well as INAD and TRP to DRM raft fractions (Fig. 4). These results suggest that the INAD-TRP interaction, and possibly the rhabdomeric localization of complexes conferred by this interaction, is required for the translocation of components to DRM rafts.

Recruitment of INAD-Signaling Complexes to Lipid Rafts Requires Activation of the Entire Phototransduction Cascade

To examine the signaling pathway required for triggering the light-induced translocation of INAD-signaling complexes to lipid rafts, we first examined the requirement for components of the phototransduction pathway. We isolated head membranes from dark-raised and light-exposed mutant strains deficient for the major rhodopsin Rh1 (*ninaE*), G_qα (*dgg*), PLC (*norpA*), TRP (*trp*), and eye-PKC (*inaC*). Mutant membranes were subjected to cold detergent treatment and subsequent floatation through density gradients. To test for the recruitment of INAD-signaling complexes to DRM fractions, we probed immunoblots of density gradient fractions for the INAD scaffold protein, one partner in the complex, eye-PKC and/or PLC, and one protein not in the complex, G_qα. In wild-type, INAD and eye-PKC were both recruited to lipid raft fractions with light-exposure, while G_qα remained solubilized in the higher density non-raft fractions in both dark and light-exposed membranes, as expected (Figs. 1,6). In contrast, in all of the phototransduction mutants tested, INAD, eye-PKC, and G_qα showed no shift to DRM raft fractions with light-exposure (Fig. 5). These results show that the light-induced mobilization of INAD-signaling complexes to lipid rafts requires Rh1, G_qα, PLC, TRP, and eye-PKC, suggesting that activation of the entire phototransduction cascade is essential for triggering the recruitment to lipid rafts.

To confirm that ultimately it is the stimulation of the light-activated channels that is required for triggering the translocation of INAD-signaling complexes to DRM rafts, we used *retinal degeneration-A* (*rdgA*) mutants. Null mutants of the *rdgA* gene, which encodes diacylglycerol kinase (DGK), have been shown to display constitutive activation of TRP and TRPL channels (Raghu et al., 2000) and, consequently, severe retinal degeneration even in newly-eclosed flies (Harris and Stark, 1977; Hotta and Benzer, 1970; Johnson et al., 1982; Matsumoto et al., 1988). Instead, we used the *rdgA³* allele, which contains a hypomorphic mutation resulting in less, but significant, constitutive activation of TRP and TRPL channels (Hardie et al., 2002; Raghu et al., 2000) and no degeneration at eclosion (Georgiev et al., 2005). Since *rdgA³* mutants were found to degenerate over 72 hours following eclosion (Georgiev et al., 2005), we collected flies which were less than 6 hours old to minimize effects due to degeneration. We then examined whether signaling components were associated with DRM rafts in these dark-raised *rdgA³* mutants. Strikingly, we found that INAD, PLC, TRP, and eye-PKC were all associated with DRM fractions in dark-raised *rdgA³* mutants (Fig. 6). G_qα and Rh1 remained in heavier density fractions similar to wild-type. These results suggest that activation of TRP and TRPL channels is sufficient to trigger the translocation of INAD-signaling complexes to DRM rafts.

Phosphorylated Eye-PKC is Selectively Recruited to Lipid Rafts

What is the role of lipid rafts in photoreceptors? One possibility is that local translocation of INAD-signaling complexes to these micro-domains contributes to the regulation of signaling. If DRM rafts are indeed micro-signaling platforms, then we would expect them to recruit mature components, enabled for immediate signaling. If, on the other hand, lipid rafts function in the targeting or trafficking of signaling proteins, we might expect to see selective recruitment of immature signaling components. To test whether mature versus immature components of INAD-signaling complexes are selectively recruited to DRM rafts, we chose to examine the form of eye-PKC associated with DRM rafts. Previous studies have shown that immature and mature forms of PKC are marked by the phosphorylation state of the kinase. Immature PKC is unphosphorylated, while mature PKC is phosphorylated multiple times, making it available for activation by Ca^{2+} and DAG (Newton, 2003). We investigated whether the phosphorylated form of eye-PKC (P-eye-PKC) is selectively recruited to DRM rafts upon light-exposure. First, using an antibody that does not distinguish between the phosphorylation states of eye-PKC (courtesy of C.S. Zuker, Smith et al., 1991a), we examined immunoblots of density gradient fractions from light-exposed wild-type flies in more detail (Fig. 7). Two bands of slightly different molecular weights are recognized by this antibody, both of which have been confirmed to be absent in *inaC* (eye-PKC) null mutants (data not shown). Both eye-PKC forms appeared to be present in the solubilized heavier density fraction #4, while primarily the higher molecular weight eye-PKC form is present in the light density fraction #2 (Fig. 7), suggesting that P-eye-PKC was selectively recruited to DRM rafts, while immature eye-PKC is present primarily in non-raft fractions #4–5. To confirm that it was the P-eye-PKC form that was indeed recruited to DRM fractions, we used a phospho-specific antibody that recognizes only the phosphorylated form of PKC (courtesy of A. Newton; Dutil et al., 1998). We found that indeed P-eye-PKC was primarily present in the DRM raft fraction #2 (Fig. 7). These results suggest that INAD-signaling complexes recruited to DRM rafts in photoreceptors contain only the mature, activatable form of eye-PKC, suggesting that DRM rafts in photoreceptors are likely to function in a signaling, versus trafficking, capacity.

Discussion

Although much work has been performed on the organization of phototransduction components in *Drosophila* as a model for G-protein coupled signaling cascades, this is the first study implicating the involvement of DRM rafts. We report the light-induced recruitment of INAD-signaling complexes to DRM rafts in the signaling compartment –the rhabdomere– of photoreceptors. We show that translocation to DRM domains is dependent on the PDZ4 and PDZ5 domains of INAD, the INAD-TRP interaction, and perhaps the rhabdomeric localization of components. Genetic analysis also demonstrates that activation of the entire phototransduction cascade is required for the translocation of INAD-signaling complexes to DRM rafts. Finally, selective recruitment of the mature phosphorylated form of eye-PKC to DRM rafts suggests that DRM domains are likely to function in a signaling capacity.

Local Translocation to Lipid Rafts Versus Subcellular Translocation of Signaling Components

Recent studies have revealed that some phototransduction components in *Drosophila* and vertebrate photoreceptors undergo a light-induced translocation between the rhabdomere and cell body (Bahner et al., 2002; Cronin et al., 2004; Cronin et al., 2006; Kosloff et al., 2003; Lee and Montell, 2004; Lee et al., 2003; Sokolov et al., 2002). The subcellular translocation of these components has been proposed to contribute to long-term light adaptation (Bahner et al., 2002; Cronin et al., 2004; Lee et al., 2003; Sokolov et al., 2002). While light appears to regulate the quantity of $\text{G}_q\alpha$, TRPL, and arrestin-2 protein available for signaling in the rhabdomere, no light-induced changes in subcellular localization have been observed for any

of the components of the INAD-signaling complex. In this report, we show instead that components of the INAD-signaling complex undergo light-regulated translocation to DRM rafts within the rhabdomeres of photoreceptors. While subcellular translocation of components out of the rhabdomere may regulate the overall level of protein available for signaling, local translocation of components to lipid raft micro-domains is likely to regulate more immediate signaling mechanisms. Although two hours of light-exposure were used in this study to induce a signal that was robust and reliable enough to be observed by the biochemical assay used, future studies, perhaps using single fluorophore tracking microscopy (Kusumi et al., 2005; Schutz et al., 2000), will need to examine real-time translocation of components to DRM rafts.

To examine the role lipid rafts play in signaling, we performed electroretinogram (ERG) recordings on *ergΔ*-fed flies (see Fig. 2). We found no apparent differences from flies fed standard fly food. We suspect, however, that signaling defects are likely to be missed in such a gross extracellular recording. Future whole-cell voltage-clamp recordings from single raft-depleted photoreceptor cells will be more informative. This, however, is not yet feasible with one-month old adult *ergΔ*-fed flies since whole-cell recording has only been successful with pupae or newly-eclosed flies. Given that flies are unable to develop to eclosion on the *ergΔ* food, an alternate ergosterol depletion method will need to be developed for these studies.

How might lipid rafts regulate signaling?

The recruitment of INAD-signaling complexes to DRM raft domains may serve to further concentrate components, increasing the rate of protein-protein interactions during signaling as well as increasing the levels of local second messengers created, possibly enhancing the speed and/or amplitude of the light-response. Another possibility is that lipid rafts serve as micro-environments that protect or isolate signaling components from positive or negative regulators present in non-raft domains. For instance, translocation of PLC to lipid rafts may serve to isolate PLC from further activation by $G_q\alpha$, contributing to deactivation of the light-response.

Micro-domains created by lipid rafts may also provide a special micro-environment that regulates signaling components. For example, TRP channels may have altered activation or deactivation properties in different lipid environments. Indeed, other ion channels have been shown to display different biophysical properties depending on whether they are associated with DRM rafts or not (Ambudkar et al., 2004; Martens et al., 2004; O'Connell et al., 2004; Szabo et al., 2004; Tillman and Cascio, 2003). Although it is well established that activation of the effector PLC is essential for activation of TRP and TRPL channels, it is still uncertain what element(s) downstream of PLC are responsible for directly gating the channels. Recent reports have suggested that the channels are activated by poly-unsaturated fatty acids derived from the second messenger DAG (Chyb et al., 1999; Hardie et al., 2002; Raghu et al., 2000) and that maintaining PIP_2 levels is necessary for sustained light responsiveness (Hardie et al., 2001). Thus, the heterogeneous distribution of lipids present in raft and non-raft micro-domains may indeed differentially regulate TRP channels. For example, PIP_2 has been reported to accumulate in DRM raft domains of several cell types (Caroni, 2001; Hope and Pike, 1996; Liu et al., 1998; Pike and Casey, 1996; Pike and Miller, 1998) and raft disruption has been shown to mislocalize PIP_2 and affect phosphatidylinositol turnover (Pike and Miller, 1998). Such a concentrated pool of PIP_2 , if present in lipid rafts of *Drosophila* photoreceptors, may indeed play a role in the activation of TRP channels when PLC and TRP are translocated to lipid rafts. Since some studies have reported that application of exogenous poly-unsaturated fatty acids causes the replacement of saturated fatty acids in the membrane with unsaturated ones, leading to disruption of lipid raft domains (Simons and Toomre, 2000; Webb et al., 2000), future studies examining the gating of TRP channels by poly-unsaturated fatty acids will now need to consider the possible role of lipid rafts in signaling.

In this study, we show that light induces the translocation of INAD-signaling complexes to DRM rafts in the rhabdomere, highlighting another facet of the dynamic nature of signaling components in photoreceptors. While subcellular movements of proteins are likely to contribute to long-term light adaptation, local translocation to micro-domains within the rhabdomere are likely to modulate more immediate signaling mechanisms. Future studies are likely to investigate the function of lipid rafts in activation/deactivation of PLC, gating of TRP channels, as well as light-adaptation. Using *Drosophila* as a model system offers the opportunity to combine biochemical studies with *Drosophila* genetics to identify the function of lipid rafts *in vivo*.

Experimental Methods

Fly Stocks

The following *Drosophila* stocks were used: *w¹¹¹⁸* as wild type, *inaD¹* (Tsunoda et al., 1997), *norpA^{P41}* (Bloomquist et al., 1988), *trp^{P343}* (Scott et al., 1997), *inaC^{P209}* (Smith et al., 1991a), *dgg¹* (Scott et al., 1995), *ninaE¹¹⁷* (O'Tousa et al., 1985), *rdgA³* (Harris and Stark, 1977; Johnson et al., 1982), *w¹¹¹⁸; cn inaD¹ bw*; *inaD^{PDZ3}/TM6B* (Tsunoda et al., 2001), *w¹¹¹⁸; cn trp^{C34} bw*; *trp³⁴³* (Tsunoda et al., 2001), and *w¹¹¹⁸; cn inaD¹ bw*; *inaD^{PDZ123-1D4}*. To generate the *inaD^{PDZ123-1D4}* construct, oligonucleotide primers were designed to amplify the truncated *inaD^{PDZ123}* and fuse it to the epitope tag 1D4, encoding 6 amino acids from the C-terminus of bovine rhodopsin. The *inaD^{PDZ123-1D4}* construct was subcloned into a P-element mediated transformation vector containing five Glass-binding sites derived from the *ninaE* promoter (pGMR; Hay et al., 1994). To generate transgenic fly lines, we performed P-element mediated transformations by standard techniques.

Light/Dark Exposure

All flies were reared in a 25°C dark incubator and collected weekly, except for *inaD¹*, *ninaE¹¹⁷*, and *trp^{C34}* flies which were collected daily (≤ 24 hours). Light-exposed flies were placed 15 cm from a white light source (Lambda LS 175W Xenon arc lamp with 400–700 nm bandpass filter; Sutter Instruments, Novato, CA, or equivalent) for two hours at a light intensity of $\sim 57 \times 10^3$ lux. Two hours of light-exposure were used in order to generate a robust signal that could be reliably detected by the biochemical assay used (below). All experiments were conducted at room temperature. After illumination, flies were immediately frozen in liquid nitrogen. Dark-raised flies were collected under dim red light and frozen similarly.

Membrane Isolation

Frozen adult fly heads were isolated and homogenized in a chilled glass-glass homogenizer in ice-cold homogenization buffer A (30 mM NaCl, 20 mM HEPES, 5 mM EDTA, pH 7.5); 1 g fly heads/1ml buffer. The homogenate was centrifuged at 5000 rpm for 2 minutes at 4°C to remove chitinous material. The supernatant was saved on ice. To extract any membranes trapped in the chitinous pellet, the chiton pellet was re-homogenized in a half-volume ice-cold buffer A and spun as described above; membranes were extracted from the chiton pellet twice. The three supernatant extracts were combined and spun at 55,000 rpm for 30 minutes at 4°C (Beckman ultracentrifuge, Optima TLX, TLA100-4 rotor) to isolate membranes. The membrane pellet was re-homogenized in chilled buffer A. Protein content was quantitated using the DC protein assay (Bio-Rad, Hercules, CA).

Floation through Optiprep Density Gradients

Membranes (1300 μ g total protein) were solubilized in 1% Triton X-100 in a total volume of 250 μ l for 1 hour on ice unless otherwise stated. 500 μ l of 60% Optiprep was added to the solubilized membranes, bringing the sample to a 40% Optiprep concentration. This sample

was placed at the bottom of an ultra-clear Beckman ultracentrifuge tube and overlaid with 1.2 mL 30% Optiprep (in buffer A, 1% Triton X-100) followed by 200 μ L of 1% Triton X-100 in buffer A. The sample was centrifuged in a TLS-55 Beckman “hanging bucket” rotor at 55,000 rpm for 2 hours at 4°C. Six fractions (350 μ L) were collected, from top (#1) to bottom (#6). 20 μ L of each fraction (unless otherwise stated) were run on 10% polyacrylamide gels by standard SDS-PAGE techniques and subjected to standard immunoblot analysis using antibodies against INAD (1:1000), TRP (1:200), eye-PKC (1:1000), PLC (1:3000), G α (1:400), Rh1 (1:100), phospho-PKC (P500; 1:500), and 1D4 (1:100). Antibodies against INAD, TRP, eye-PKC, PLC, Rh1, and 1D4 were received as a gift from C.S. Zuker (University of California, San Diego, CA), P500 was a gift from A. Newton (University of California, San Diego, CA). All incubations were conducted overnight at room temperature.

Regular Laboratory Fly Food and Mutant Yeast Fly Food

Standard laboratory fly food was used for all fly stocks unless otherwise stated. To make standard fly food, 312 g yeast and 756 g cornmeal were mixed with 2 L of water until the mixture was homogenous. This mixture, 112 g agar, and 756 mL molasses were added to 3.8 L water, heated to 80°C, and cooked until the food reached 90°C. 2 L of cold water was then added to the mixture and the food was permitted to cool to 70°C followed by the addition of 80 mL propionic acid and 231 mL methyl 4-hydroxybenzoate (Sigma).

For *ergA* mutant yeast fly food, *erg2A* (RH2897), *erg6A* (RH3622) and *erg2Aerg6A* (RH3616) mutant yeast strains, which were received as a gift from Dr. H. Riezman (Biozentrum of the University of Basel, Basel, Switzerland), were used. YPUAD medium (2% Glucose, 1% Yeast extract, 2% Peptone, 40 mg/L each of Adenine, Uracil, and Tryptophan, pH 6.5) was autoclaved for 15 minutes and used to culture the mutant strains. Mutant colonies for all three strains were selected, dissolved in 1 mL water and added to 50 mL YPUAD medium and cultured on an orbital shaker at 200 rpm, overnight at 28°C. Cultures were centrifuged at 1000 G for 15 minutes. Yeast pellets were washed two times with water, suspended in 30 mL water, and centrifuged again at 1000 G for 15 minutes. Yeast pellets were stored at 4°C. To cook mutant yeast fly food, 1 gram agar, 5 grams glucose, and 50 mL water were heated together to 100°C. 5 grams of mutant yeast (pellets) were added and the mixture continued to be heated until homogeneous. When the food cooled to 70°C, 0.5 mL methyl 4-hydroxybenzoate (Sigma) was added to the food to prevent mold growth. The food was then poured into polypropylene bottles and allowed to cool overnight before using.

Sterol Extraction and Ultraviolet Spectral Analysis

Total sterols were extracted from whole fly membranes as follows. For each sample, 50 flies were collected and homogenized in 160 μ L distilled water. The homogenate was centrifuged at 6000 rpm for 4 minutes and the supernatant collected. The pellet was then resuspended in a half-volume of water and spun as described above. The supernatants were combined and stored at -80 °C prior to analysis. Protein content was quantitated as described above. Protocol for sterol extraction was adapted from Arthington-Skaggs et al. (1999) (Arthington-Skaggs et al., 1999). Membranes (1260.36 μ g total protein) were transferred to a glass tube and 3 mL 25% alcoholic potassium hydroxide solution (25 g of KOH and 35 mL of distilled water, brought to 100 mL with 100% ethanol) was added, followed by 1 minute of vortexing. Samples were incubated at 82 °C for 1 hour, then allowed to cool to room temperature. Sterols were extracted by addition of 1 mL distilled water and 3 mL of *n*-heptane (Sigma), followed by vigorous mixing with glass Pasteur pipettes. The upper heptane layer was transferred to a new glass tube and samples were dried under a stream of nitrogen while being warmed at 30°C (Reacti-Therm Heating Module, Pierce). Dried samples were resuspended in 200 μ L of 100% ETOH. For detection of ergosterol, samples were scanned spectrophotometrically between 250 and 300 nm (Pharma Spec UV-1700, UV-visible spectrophotometer, Shimadzu). To quantitate

ergosterol content, absorbance readings were taken at 281.5 nm. Differences between average absorbance readings were analyzed using Student's T-test, using P values <0.05.

Immunostaining of Retinal Tissue Sections

Flies were either light-exposed or dark-raised (as described above). Heads were fixed in 3% paraformaldehyde in PBS for 1 hour on ice, followed by several washes with PBS, and infiltration with 2.3 M sucrose in PBS overnight at 4°C. Heads were then bisected, positioned on ultramicrotomy pins (Ted Pella, Redding, CA) and frozen in liquid nitrogen. 1 µm thick sections were cut using a Leica Ultracut UCT attached to an EM FCS cryo unit (Leica Microscopy and Scientific Instruments Group, Heerbrugg, Switzerland) at -81°C. Retinal sections were incubated in blocking solution (1% BSA and 0.1% saponin in PBS) for at least 1 hour at room temperature followed by overnight incubation with anti-INAD or anti-1D4 antibody (1:1000 or 1:100, respectively, in blocking solution) at 4°C. After four washes (0.1% saponin in PBS), rhodamine-conjugated goat-anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA) was used at 1:200 for 1 hour at room temperature. Slides were mounted with 90% glycerol and p-phenylenediamine (Sigma Aldrich, St. Louis, MO). For filipin (Sigma) staining, retinal sections were incubated in 50 µg/mL filipin overnight at 4°C and then washed with PBS and mounted as described above.

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References

- Adamski FM, Zhu MY, Bahiraei F, Shieh BH. Interaction of Eye Protein Kinase C and INAD in *Drosophila*. *Journal of Biological Chemistry* 1998;273:17713–17719. [PubMed: 9651370]
- Ambudkar IS, Brazer SC, Liu X, Lockwich T, Singh B. Plasma membrane localization of TRPC channels: role of caveolar lipid rafts. *Novartis Found Symp* 2004;258:63–70. [PubMed: 15104176]discussion 70–4, 98–102, 263–6
- Arthington-Skaggs BA, Jradi H, Desai T, Morrison CJ. Quantitation of ergosterol content: novel method for determination of fluconazole susceptibility of *Candida albicans*. *J Clin Microbiol* 1999;37:3332–7. [PubMed: 10488201]
- Bagnat M, Simons K. Cell surface polarization during yeast mating. *Proc Natl Acad Sci U S A* 2002;99:14183–8. [PubMed: 12374868]
- Bahner M, Frechter S, Da Silva N, Minke B, Paulsen R, Huber A. Light-regulated subcellular translocation of *Drosophila* TRPL channels induces long-term adaptation and modifies the light-induced current. *Neuron* 2002;34:83–93. [PubMed: 11931743]
- Bloomquist B, Shortridge R, Schneuwly S, Perdew M, Montell C, Steller H, Rubin G, Pak W. Isolation of a Putative Phospholipase C gene of *Drosophila*, *norpA*, and its role in phototransduction. *Cell* 1988;54:723–733. [PubMed: 2457447]
- Brown DA, London E. Structure of detergent-resistant membrane domains: does phase separation occur in biological membranes? *Biochem Biophys Res Commun* 1997;240:1–7. [PubMed: 9367871]
- Brown DA, London E. Functions of lipid rafts in biological membranes. *Annu Rev Cell Dev Biol* 1998;14:111–36. [PubMed: 9891780]
- Brown DA, London E. Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J Biol Chem* 2000;275:17221–4. [PubMed: 10770957]
- Brown DA, Rose JK. Sorting of GPI-anchored proteins to glycolipid- enriched membrane subdomains during transport to the apical cell surface. *Cell* 1992;68:533–44. [PubMed: 1531449]
- Caroni P. New EMBO members' review: actin cytoskeleton regulation through modulation of PI(4,5)P (2) rafts. *Embo J* 2001;20:4332–6. [PubMed: 11500359]

- Chyb S, Raghu P, Hardie RC. Polyunsaturated fatty acids activate the *Drosophila* light-sensitive channels TRP and TRPL. *Nature* 1999;397:255–9. [PubMed: 9930700]
- Cronin M, Diao F, Tsunoda S. The Light-Dependent Subcellular Translocation of Gqa in *Drosophila* Photoreceptors is Facilitated by the Photoreceptor-Specific Myosin III, NINAC. *Journal of Cell Science* 2004;117:4797–4806. [PubMed: 15340015]
- Cronin MA, Lieu MH, Tsunoda S. Two Stages of Light-Dependent TRPL-Channel Translocation in *Drosophila* Photoreceptors. *Journal of Cell Science* 2006;119:2935–2944. [PubMed: 16787936]
- Dutil EM, Toker A, Newton AC. Regulation of conventional protein kinase C isozymes by phosphoinositide-dependent kinase 1 (PDK-1). *Curr Biol* 1998;8:1366–75. [PubMed: 9889098]
- Eisenkolb M, Zenzmaier C, Leitner E, Schneiter R. A specific structural requirement for ergosterol in long-chain fatty acid synthesis mutants important for maintaining raft domains in yeast. *Mol Biol Cell* 2002;13:4414–28. [PubMed: 12475962]
- Eroglu C, Brugger B, Wieland F, Sinning I. Glutamate-binding affinity of *Drosophila* metabotropic glutamate receptor is modulated by association with lipid rafts. *Proc Natl Acad Sci U S A* 2003;100:10219–24. [PubMed: 12923296]
- Georgiev P, Garcia-Murillas I, Ulahannan D, Hardie RC, Raghu P. Functional INAD complexes are required to mediate degeneration in photoreceptors of the *Drosophila* rdgA mutant. *J Cell Sci* 2005;118:1373–84. [PubMed: 15755798]
- Golub T, Wacha S, Caroni P. Spatial and temporal control of signaling through lipid rafts. *Curr Opin Neurobiol* 2004;14:542–50. [PubMed: 15464886]
- Harder T, Simons K. Caveolae, DIGs, and the dynamics of sphingolipid- cholesterol microdomains. *Curr Opin Cell Biol* 1997;9:534–42. [PubMed: 9261060]
- Hardie RC, Martin F, Cochrane GW, Juusola M, Georgiev P, Raghu P. Molecular basis of amplification in *Drosophila* phototransduction: roles for G protein, phospholipase C, and diacylglycerol kinase. *Neuron* 2002;36:689–701. [PubMed: 12441057]
- Hardie RC, Raghu P. Visual transduction in *Drosophila*. *Nature* 2001;413:186–93. [PubMed: 11557987]
- Hardie RC, Raghu P, Moore S, Juusola M, Baines RA, Sweeney ST. Calcium influx via TRP channels is required to maintain PIP2 levels in *Drosophila* photoreceptors. *Neuron* 2001;30:149–59. [PubMed: 11343651]
- Harris WA, Stark WS. Hereditary retinal degeneration in *Drosophila melanogaster*. A mutant defect associated with the phototransduction process. *J Gen Physiol* 1977;69:261–91. [PubMed: 139462]
- Hay BA, Wolff T, Rubin GM. Expression of baculovirus P35 prevents cell death in *Drosophila*. *Development* 1994;120:2121–2129. [PubMed: 7925015]
- Hoehne M, de Couet HG, Stuermer CA, Fischbach KF. Loss- and gain- of-function analysis of the lipid raft proteins Reggie/Flotillin in *Drosophila*: they are posttranslationally regulated, and misexpression interferes with wing and eye development. *Mol Cell Neurosci* 2005;30:326–38. [PubMed: 16154361]
- Hooper NM. Detergent-insoluble glycosphingolipid/cholesterol-rich membrane domains, lipid rafts and caveolae (review). *Mol Membr Biol* 1999;16:145–56. [PubMed: 10417979]
- Hope HR, Pike LJ. Phosphoinositides and phosphoinositide-utilizing enzymes in detergent-insoluble lipid domains. *Mol Biol Cell* 1996;7:843–51. [PubMed: 8816992]
- Hotta Y, Benzer S. Genetic dissection of the *Drosophila* nervous system by means of mosaics. *Proceedings of the National Academy of Sciences of the United States of America* 1970;67:1156–1163. [PubMed: 5274445]
- Huber A. Scaffolding proteins organize multimolecular protein complexes for sensory signal transduction. *Eur J Neurosci* 2001;14:769–76. [PubMed: 11576180]
- Johnson M, Frayer K, Stark W. Characteristics of rdgA: mutants with retinal degeneration in *Drosophila*. *J Insect Physiology* 1982;28:233–242.
- Keesler GA, Casey WM, Parks LW. Stimulation by heme of steryl ester synthase and aerobic sterol exclusion in the yeast *Saccharomyces cerevisiae*. *Arch Biochem Biophys* 1992;296:474–81. [PubMed: 1632640]
- Kosloff M, Elia N, Joel-Almagor T, Timberg R, Zars TD, Hyde DR, Minke B, Selinger Z. Regulation of light-dependent Gqalpha translocation and morphological changes in fly photoreceptors. *Embo J* 2003;22:459–68. [PubMed: 12554647]

- Kusumi A, Ike H, Nakada C, Murase K, Fujiwara T. Single-molecule tracking of membrane molecules: plasma membrane compartmentalization and dynamic assembly of raft-philic signaling molecules. *Semin Immunol* 2005;17:3–21. [PubMed: 15582485]
- Lai EC. Lipid rafts make for slippery platforms. *J Cell Biol* 2003;162:365–70. [PubMed: 12885764]
- Lee SJ, Montell C. Light-dependent translocation of visual arrestin regulated by the NINAC myosin III. *Neuron* 2004;43:95–103. [PubMed: 15233920]
- Lee SJ, Xu H, Kang LW, Amzel LM, Montell C. Light adaptation through phosphoinositide-regulated translocation of *Drosophila* visual arrestin. *Neuron* 2003;39:121–32. [PubMed: 12848937]
- Liu Y, Casey L, Pike LJ. Compartmentalization of phosphatidylinositol 4,5-bisphosphate in low-density membrane domains in the absence of caveolin. *Biochem Biophys Res Commun* 1998;245:684–90. [PubMed: 9588175]
- Martens JR, O'Connell K, Tamkun M. Targeting of ion channels to membrane microdomains: localization of KV channels to lipid rafts. *Trends Pharmacol Sci* 2004;25:16–21. [PubMed: 14723974]
- Matsumoto E, Hirokawa K, Takagawa K, Hotta Y. Structure of retinal cells in a *Drosophila melanogaster* visual mutant, *rdgA*, at early stages of degeneration. *Cell Tissue Research* 1988;252:293–300. [PubMed: 3133115]
- Munn AL, Heese-Peck A, Stevenson BJ, Pichler H, Riezman H. Specific sterols required for the internalization step of endocytosis in yeast. *Mol Biol Cell* 1999;10:3943–57. [PubMed: 10564282]
- Nair KS, Balasubramanian N, Slepak VZ. Signal-dependent translocation of transducin, RGS9-1-Gbeta5L complex, and arrestin to detergent-resistant membrane rafts in photoreceptors. *Curr Biol* 2002;12:421–5. [PubMed: 11882295]
- Newton AC. Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. *Biochem J* 2003;370:361–71. [PubMed: 12495431]
- Norman AW, Demel RA, de Kruffy B, van Deenen LL. Studies on the biological properties of polyene antibiotics. Evidence for the direct interaction of filipin with cholesterol. *J Biol Chem* 1972;247:1918–29. [PubMed: 5012767]
- O'Connell KM, Martens JR, Tamkun MM. Localization of ion channels to lipid Raft domains within the cardiovascular system. *Trends Cardiovasc Med* 2004;14:37–42. [PubMed: 15030787]
- O'Tousa JE, Baehr W, Martin RL, Hirsh J, Pak WL, Applebury ML. The *Drosophila ninaE* gene encodes an opsin. *Cell* 1985;40:839–850. [PubMed: 2985266]
- Pike LJ, Casey L. Localization and turnover of phosphatidylinositol 4,5- bisphosphate in caveolin-enriched membrane domains. *J Biol Chem* 1996;271:26453–6. [PubMed: 8900109]
- Pike LJ, Miller JM. Cholesterol depletion delocalizes phosphatidylinositol bisphosphate and inhibits hormone-stimulated phosphatidylinositol turnover. *J Biol Chem* 1998;273:22298–304. [PubMed: 9712847]
- Raghu P, Usher K, Jonas S, Chyb S, Polyanovsky A, Hardie RC. Constitutive activity of the light-sensitive channels TRP and TRPL in the *Drosophila* diacylglycerol kinase mutant, *rdgA*. *Neuron* 2000;26:169–79. [PubMed: 10798401]
- Ranganathan R, Malicki DM, Zuker CS. Signal Transduction in *Drosophila* Photoreceptors. *Annual Review of Neuroscience* 1995a;18:283–317.
- Ranganathan R, Ross EM. PDZ domain proteins: scaffolds for signaling complexes. *Curr Biol* 1997;7:R770–3. [PubMed: 9382826]
- Rietveld A, Neutz S, Simons K, Eaton S. Association of sterol- and glycosylphosphatidylinositol-linked proteins with *Drosophila* raft lipid microdomains. *J Biol Chem* 1999;274:12049–54. [PubMed: 10207028]
- Sankaram MB, Thompson TE. Interaction of cholesterol with various glycerophospholipids and sphingomyelin. *Biochemistry* 1990;29:10670–5. [PubMed: 2176878]
- Schroeder R, London E, Brown D. Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins: GPI-anchored proteins in liposomes and cells show similar behavior. *Proc Natl Acad Sci U S A* 1994;91:12130–4. [PubMed: 7991596]

- Schutz GJ, Kada G, Pastushenko VP, Schindler H. Properties of lipid microdomains in a muscle cell membrane visualized by single molecule microscopy. *Embo J* 2000;19:892–901. [PubMed: 10698931]
- Scott K, Leslie A, Sun Y, Hardy R, Zuker C. Gq Protein Function *in vivo*: Genetic Dissection of Its Role in Photoreceptor Cell Physiology. *Neuron* 1995;15:919–927. [PubMed: 7576640]
- Scott K, Sun YM, Beckingham K, Zuker CS. Calmodulin regulation of Drosophila light-activated channels and receptor function mediates termination of the light response *in vivo*. *Cell* 1997;91:375–383. [PubMed: 9363946]
- Shieh BH, Zhu MY. Regulation of the TRP Ca²⁺ channel by INAD in Drosophila photoreceptors. *Neuron* 1996;16:991–8. [PubMed: 8630257]
- Shieh BH, Zhu MY, Lee JK, Kelly IM, Bahiraei F. Association of INAD with NORPA is essential for controlled activation and deactivation of Drosophila phototransduction *in vivo*. *Proceedings of the National Academy of Sciences of the United States of America* 1997;11:12682–7. [PubMed: 9356510]
- Simons K, Ikonen E. Functional rafts in cell membranes. *Nature* 1997;387:569–72. [PubMed: 9177342]
- Simons K, Toomre D. Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* 2000;1:31–9. [PubMed: 11413487]
- Simons K, van Meer G. Lipid sorting in epithelial cells. *Biochemistry* 1988;27:6197–202. [PubMed: 3064805]
- Smith DP, Ranganathan R, Hardy RW, Marx J, Tsuchida T, Zuker CS. Photoreceptor deactivation and retinal degeneration mediated by a photoreceptor-specific protein kinase C. *Science* 1991a; 254:1478–1484. [PubMed: 1962207]
- Sokolov M, Lyubarsky AL, Strissel KJ, Savchenko AB, Govardovskii VI, Pugh EN Jr, Arshavsky VY. Massive light-driven translocation of transducin between the two major compartments of rod cells: a novel mechanism of light adaptation. *Neuron* 2002;34:95–106. [PubMed: 11931744]
- Szabo I, Adams C, Gulbins E. Ion channels and membrane rafts in apoptosis. *Pflügers Arch* 2004;448:304–12. [PubMed: 15071744]
- Takeda T, Chang F. Role of fission yeast myosin I in organization of sterol rich membrane domains. *Curr Biol* 2005;15:1331–6. [PubMed: 16051179]
- Tillman TS, Cascio M. Effects of membrane lipids on ion channel structure and function. *Cell Biochem Biophys* 2003;38:161–90. [PubMed: 12777713]
- Trocha PJ, Sprinson DB. Location and regulation of early enzymes of sterol biosynthesis in yeast. *Arch Biochem Biophys* 1976;174:45–51. [PubMed: 7207]
- Tsunoda S, Sierralta J, Sun Y, Bodner R, Suzuki E, Becker A, Socolich M, Zuker CS. A multivalent PDZ-domain protein assembles signalling complexes in a G-protein-coupled cascade. *Nature* 1997;388:243–9. [PubMed: 9230432]
- Tsunoda S, Sierralta J, Zuker CS. Specificity in signaling pathways: assembly into multimolecular signaling complexes. *Current Opinion in Genetics and Development* 1998;8:419–422. [PubMed: 9729717]
- Tsunoda S, Sun Y, Suzuki E, Zuker C. Independent anchoring and assembly mechanisms of INAD signaling complexes in Drosophila photoreceptors. *J Neurosci* 2001;21:150–8. [PubMed: 11150331]
- Tsunoda S, Zuker CS. The organization of INAD-signaling complexes by a multivalent PDZ domain protein in Drosophila photoreceptor cells ensures sensitivity and speed of signaling. *Cell Calcium* 1999;26:165–71. [PubMed: 10643554]
- van Huizen R, Miller K, Chen DM, Li Y, Lai ZC, Raab RW, Stark WS, Shortridge RD, Li M. Two distantly positioned PDZ domains mediate multivalent INAD-phospholipase C interactions essential for G protein-coupled signaling. *EMBO Journal* 1998;17:2285–2297. [PubMed: 9545241]
- Wachtler V, Rajagopalan S, Balasubramanian MK. Sterol-rich plasma membrane domains in the fission yeast *Schizosaccharomyces pombe*. *J Cell Sci* 2003;116:867–74. [PubMed: 12571284]
- Webb Y, Hermida-Matsumoto L, Resh MD. Inhibition of protein palmitoylation, raft localization, and T cell signaling by 2-bromopalmitate and polyunsaturated fatty acids. *J Biol Chem* 2000;275:261–70. [PubMed: 10617614]
- Xu X, Bittman R, Duportail G, Heissler D, Vilcheze C, London E. Effect of the structure of natural sterols and sphingolipids on the formation of ordered sphingolipid/sterol domains (rafts). *Comparison of*

cholesterol to plant, fungal, and disease-associated sterols and comparison of sphingomyelin, cerebroside, and ceramide. *J Biol Chem* 2001;276:33540–6. [PubMed: 11432870]

Zhai L, Chaturvedi D, Cumberledge S. *Drosophila* wnt-1 undergoes a hydrophobic modification and is targeted to lipid rafts, a process that requires porcupine. *J Biol Chem* 2004;279:33220–7. [PubMed: 15166250]

Fig. 1. Light-Induced Recruitment of INAD-Signaling Complexes to Lipid Rafts. *Top and Middle*, Membranes from either dark-raised or light-exposed wild-type flies were treated with 1% Triton X-100 at 4°C, followed by floatation through Optiprep density gradients (see Experimental Methods). Shown are representative immunoblots of the six fractions collected from top (1) to bottom (6) of density gradients. In dark-raised flies (Dark), all phototransduction components examined were found primarily in heavier density fractions #4–6. With light-exposure (Light; see Experimental Methods), INAD, and its target proteins, PLC, TRP, and eye-PKC, were recruited to detergent-resistant light density fractions (#1–2). In contrast, Rh1 and Gqα from membranes of both dark-raised and light-exposed flies were solubilized and

found primarily in fractions #5–6. *Bottom*, Membranes from light-exposed wild-type flies were treated with 1% Triton X-100 at 37°C and assayed in similar Optiprep density floatation gradients. Shown is a representative immunoblot of density gradient fractions. Recruitment of signaling components to light density fractions is almost completely abolished with detergent treatment at 37°C, consistent with the proposal that INAD-signaling complex components are indeed translocating to DRM raft domains.

Fig. 2.

Disruption of INAD-Signaling Complex Recruitment to DRM Rafts when Dietary Ergosterol is Limited. (A) Wild-type flies were fed food containing wild-type, *erg2Δ*, *erg6Δ*, or *erg2Δerg6Δ* yeast extract (see Experimental Methods), as indicated, for one month. Sterols were extracted from whole fly membranes, and then scanned spectrophotometrically. Shown are representative spectral profiles (250 nm – 300 nm) of sterols from flies fed on these different fly foods; this four-peaked profile is characteristic of ergosterol. Flies raised on *erg2Δ*, *erg6Δ* or *erg2Δerg6Δ* mutant yeast food consistently displayed reduced absorption as compared to wild-type flies fed on standard fly food. (B) Quantitation of ergosterol absorbance. Single absorbance readings were taken at 281.5 nm for each of the sterol extract samples listed in (A). Values shown are expressed as the relative absorbance, normalized to the wild-type average absorbance readings at 281.5 nm. Flies raised on *ergΔ* mutant yeast food contained significantly reduced absorbance readings as compared to wild-type, demonstrating that flies raised on the mutant yeast do indeed contain decreased ergosterol content. (* represents a decrease in absorbance that is statistically significant from wild-type, P value <0.05). (C) Wild-type flies fed food containing wild-type, *erg2Δ*, *erg6Δ*, or *erg2Δerg6Δ* yeast extract for one month, were light-exposed and head membranes were treated with cold 1% Triton X-100, followed by floatation through Optiprep density gradients (see Experimental Methods). Shown are representative immunoblots of fractions #1–6 taken from top to bottom, respectively, of density gradients. Flies fed on wild-type food showed translocation of PLC, eye-PKC, INAD, and TRP to detergent resistant light density fractions (#1–2), as expected. In contrast, when flies were raised on *erg2Δ*, *erg6Δ* or *erg2Δerg6Δ* mutant yeast food, little to no translocation of components to DRM fractions was observed. These results suggest that limited ergosterol intake of flies results in an impairment of the DRM domains associated with INAD-signaling complex components in light-exposed flies.

Fig. 3.

Localization of Lipid Rafts in Photoreceptors. Shown are representative cross-sections of single ommatidia from dark-raised (Dark) and light-exposed (Light) wild-type flies treated with filipin or an antibody against INAD, as indicated. For reference, the rhabdomere (R) and cell body (C) of a single photoreceptor cell are indicated in one ommatidia (Dark, Filipin). Filipin staining shows that sterol-rich domains are found primarily in the rhabdomeres of photoreceptors, with no gross changes in localization induced with light-exposure. INAD is similarly localized in the rhabdomeres of photoreceptors in both dark-raised and light-exposed flies. The

Fig. 4.

INAD and TRP Mutants Reveal that PDZ4 and PDZ5 domains of INAD and the INAD-TRP Interaction are Required for Recruitment of Signaling Complexes to DRM Rafts. (A) Membranes from light-exposed wild-type, *inaD*¹ (*inaD* null mutant), *inaD*^{PDZ123-1D4}, *inaD*^{PDZ3}, and *trp*^{C34} mutants were subjected to cold detergent treatment and floatation through Optiprep density gradients (see Experimental Methods). Shown are representative immunoblots of fractions #1 to 6 taken from top to bottom, respectively, of density gradients. Light-induced translocation of eye-PKC and INAD to DRM fractions (#1–2) in wild-type (wt) are shown for reference; Gq α represents a signaling component that is not recruited to DRM raft fractions. In *inaD* null mutants, no significant translocation of TRP, PLC, or eye-PKC to

DRM fractions (#1–2) was observed, suggesting that the scaffold protein INAD is required for the recruitment of these target proteins to DRM rafts. Since even these young (<24 hours) *inaD* mutants contain somewhat lower levels of PLC, TRP, and eye-PKC, we used a larger quantity of membrane sample (1.5X). In *inaD*^{PDZ123-1D4} mutants, INAD^{PDZ123-1D4}, PLC, TRP, and eye-PKC proteins all failed to translocate to light density fractions (#1–2). In *inaD*^{PDZ3} and *trp*^{C34} mutants, the translocation of PLC, eye-PKC, INAD, and TRP to DRM fractions is significantly reduced or abolished, showing that the TRP-INAD interaction is required for the light-induced recruitment of complexes to DRM rafts. (B) Representative retinal sections (1 µm thick) from *inaD*^{PDZ123-1D4} transgenic flies (<24 hours old) were immunostained for INAD^{PDZ123-1D4} using an antibody against the epitope tag 1D4. INAD^{PDZ123-1D4} shows clear rhabdomeric localization.

Fig. 5. Recruitment of INAD-Signaling Complexes to Lipid Rafts Requires the Activation of the Entire Phototransduction Cascade. Dark-raised (Dark) and light-exposed (Light) wild-type and mutant strains deficient for Rh1 (*ninaE*^{I17}), Gqα (*dgq*¹), PLC (*norpA*^{P41}), TRP (*trp*^{P343}), and eye-PKC (*inaC*^{P209}) were analyzed for light-induced translocation of INAD, eye-PKC, and Gqα to DRM fractions (#1–2). Membranes were treated with cold 1% Triton-X 100 and floated through Optiprep density gradients; representative immunoblots of fractions #1 to 6 taken from top to bottom, respectively, are shown. In wild-type, INAD and eye-PKC displayed light-induced recruitment to light density fractions (#1–2), while Gqα remained in heavier density fractions, as expected. In contrast, in all of the mutants tested, INAD and eye-PKC failed to show any significant light-induced translocation to light density fractions (#1–2); immunoblots from dark-raised and light-exposed mutants were indistinguishable. These results suggest that activation of the entire phototransduction cascade is required for signaling translocation to DRM rafts.

Fig. 6. INAD-Signaling Complexes are Associated with DRM Rafts in Dark-Raised *rdgA*³ Mutants. Dark-raised wild-type and *rdgA*³ flies (<6 hours old) were examined for the presence of signaling components in DRM rafts. Membranes (1950 μ g total protein) were treated with cold 1% Triton-X 100 and subjected to floatation through Optiprep density gradients; representative immunoblots of fractions #1 to 6 taken from top to bottom, respectively, are shown. In dark-raised wild-type flies, all signaling proteins were primarily present in heavier density fractions, as expected. In sharp contrast, PLC, eye-PKC, INAD, and TRP were found in detergent-resistant light density fractions (#1–2) of dark-raised *rdgA*³ mutants, suggesting that constitutive activation of TRP and TRPL channels in *rdgA*³ mutants is sufficient to trigger the translocation of INAD-signaling complexes to DRM raft domains.

Fig. 7.

Phosphorylated Eye-PKC is Selectively Recruited to DRM Rafts with Light-Exposure.

Membranes from light-exposed wild-type flies were treated with cold 1% Triton-X 100 and subjected to floatation through Optiprep density gradients. Immunoblot analysis was performed on fractions from density gradients using either a polyclonal antibody against eye-PKC (top blot, PKC) that recognizes both phosphorylated (arrowhead) and un-phosphorylated forms of eye-PKC, or an antibody that recognizes only the phosphorylated form of PKC (bottom blot, P-PKC). *Top blot*, The higher molecular weight (phosphorylated) form of eye-PKC appeared to be present in the detergent-resistant light density fraction (#2) as well as in fraction #4, while the lower molecular weight (unphosphorylated) form of eye-PKC was primarily present in heavier density fractions (#4–5). *Bottom blot*, Using a phospho-specific antibody for PKC, the phosphorylated form of eye-PKC was found to be present primarily in light density fraction #2, confirming that the phosphorylated form of eye-PKC is selectively recruited to DRM fractions.