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Evidence for indirect control of phospholipase C (PLC-β) by retinoids in *Drosophila* phototransduction

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Purpose: To determine how retinoids regulate the phospholipase C (PLC) gene in the *Drosophila* visual system.
Methods: Western blotting, activity analyses and immunocytochemistry were applied to *Drosophila* reared on various diets.
Results: Western blots and activity analyses showed that retinoid deprivation decreases PLC, the product of the norpA gene, by approximately 1/3 to 1/2 in *Drosophila*. Immunocytochemistry using standard and confocal fluorescence microscopy confirmed the expectation that PLC is localized to the photoreceptive rhabdomeres. Rhabdomeres of flies that were retinoid deprived, or reared on other diets devoid of chromophore precursors, fluoresced brightly. These observations are consistent with earlier morphometric analyses showing that retinoid deprivation decreases the size of rhabdomeres. In a separate control, rhabdomeric PLC was shown to be virtually eliminated by retinoid deprivation in transgenic *Drosophila* where the norpA coding sequence was driven by the opsin promoter.
Conclusions: PLC is decreased by retinoid deprivation. Retinoid control of PLC is indirect, as expected, since the norpA promoter is so different from the promoter for rhodopsin's gene. PLC is not eliminated by deprivation but decreases in proportion to the associated decrease in rhabdomere size which, in turn, is caused by the opsin decrease. By contrast, opsin is controlled by retinoids both translationally by chromophore availability and transcriptionally. The fact that PLC is eliminated by retinoid deprivation when opsin's promoter drives the PLC gene is important evidence substantiating retinoid control via opsin's promoter.

In contrast to the vertebrate situation, retinoids, including retinoic acid, are neither essential nor toxic in *Drosophila*. This allows strategic manipulations of retinoid deprivation and replacement to study photoreceptor function. Retinoids not only serve as precursors of the light absorbing chromophore of rhodopsin, but are also involved in the regulation of expression of the apoprotein opsin [1] at the co- or post-translational level [2] and at the transcriptional level [3-5]. A *Drosophila* retinoid and fatty acid binding glycoprotein (RFABG) also shows transcriptional regulation [6].

Do retinoids regulate other vision genes? To address this issue, we used retinoid deprivation and replacement to examine regulation of phospholipase C (PLC). The PLC enzymes are central to many signal transduction systems [7]. Mutations in the *Drosophila* norpA gene, encoding a homologue of mammalian PLC-β, are PLC deficient in heads [8]. Mutants are named for the no receptor potential phenotype in the compound eye [9,10] and ocelli [11]. *Drosophila* rhodopsin activates a heterotrimeric G-protein, which, in turn, activates PLC [12]. PLC is the norpA gene product [13], localized in the rhodopsin-containing rhabdomere [14]. In the present study, we found that PLC is decreased nearly half by retinoid deprivation (as assayed by activity analyses and western blotting) but that the rhabdomeres still show bright emission in immunocytochemistry. This PLC decrease can be explained by the decrease in rhabdomere size caused by retinoid deprivation [15]. Importantly, rhabdomere fluorescence was eliminated by retinoid deprivation in a *Drosophila* stock in which opsin's promoter drives PLC, verifying the retinoid sensitivity of opsin's promoter.

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METHODS

Flies: Drosophila melanogaster were maintained on standard medium (750 ml H_2O, 8 g agar, 100 g yellow cornmeal, 40 g brewer's yeast and 100 ml molasses, with mold inhibitors), adequate for full levels of visual pigment [16,17] and visual function [17,18]. The lutein and zeaxanthin in yellow cornmeal probably provide adequate carotenoids; however, to ensure sufficient vitamin A, we supplemented our fly food with 0.125 mg/ml β-carotene, the lowest dose found to maximize visual sensitivity [17,18] and visual pigment [16,17] for retinoid deprived flies. Flies were retinoid deprived by rearing from egg to adult on Sang's medium without any retinoids [19]. Flies were reared on a 12 h light / 12 h dark (L/D) cycle of fluorescent lighting in a 25 °C incubator at an intensity of 140 lux (calibrated with a portable radiometer / photometer, Ealing model 27-5479, Holliston, MA).

To determine the effects of various retinoids and nutrients, flies were reared from egg to adult on Sang's medium containing all-trans retinoic acid (Sigma Chem. Corp., St. Louis) at 0.125 mg/ml, Beef Brain-Heart Infusion (Difco, Chicago, IL) at 37 mg/ml or β-carotene at 0.25 mg/ml. Beef Brain-Heart Infusion had been added to Sang's medium to address the following discrepency: Huber et al. [20] found that chromophore deprivation in blowflies (*Calliphora*) caused by rearing on beef heart decreased opsin without decreasing its mRNA; our thorough retinoid deprivation in *Drosophila* reared on the more defined Sang's medium decreased both [3]. Alternatively, a yeast-glucose food was made consisting of 100 g glucose, 100 g yeast, and 17 g agar in 1000 ml of water with mold inhibitor. The yeast food was used to address another discrepency: Ozaki, et al. [2] had found that a similar diet decreased opsin, indicating that it lacked chromophore precursors, but not opsin's mRNA; as stated above, Sang's medium eliminated both [3].

Carrot juice, especially useful for the rapid recovery it elicits [21,22], β-carotene, and retinoic acid were used for "replacement therapy" [3,5]. Retinoid replacement was with 10 ml of 20% sucrose in water without (control) or with 0.1 ml β-carotene or all-trans retinoic acid (0.125 mg/ml) dispersed in ethanol in the dark. Replacement was achieved by transferring deprived flies into a vial with a sliver of material from a foam stopper soaked with the fluid [15,23,24] after the flies had been put into an empty vial for 7-9 h to be certain that they would drink immediately.

The white-eyed mutant was used throughout, and this was especially important in immunocytochemistry where fluorescence of eye color pigments could have otherwise confounded the observations. For PLC activity analyses, the w^{A35} allele was used in keeping with earlier investigations [14]. The $norpA^{P24}$ mutant was used as a negative control since it is a strong allele [13,25]. Recently, a functional norpA minigene was constructed by fusing norpA cDNA to the ninaE promoter [14]. The ninaE (neither inactivation nor afterpotential) gene encodes Rh1, the opsin which is expressed in R1-6 [26,27]. Transgenic flies with this ninaE-promoter norpA-coding-sequence rescued $norpA^{P24}$ mutants, but only R1-6 receptors were rescued [14]. We used this stock to contrast retinoid regulation of PLC via the native norpA promoter vs the ectopic ninaE promoter, the latter known to be retinoid-sensitive.

Several ninaE mutants were tested also, including $ninaE^{oI17}$, a large deletion [26], and $ninaE^{ora}$, a nonsense mutation in the ninaE coding sequence [28,29]. "Ora" stands for "outer rhabdomeres absent" [30-32]. Newly-emerged $ninaE^{ora}$ flies have R1-6 rhabdomeres that diminish with age [31].

Western blotting: Procedures were published by Picking et al. [3] and detailed by Lee [33]. *Drosophila* were frozen in liquid nitrogen. Heads were separated from the bodies, ground and sonicated (Sonic Dismembrator 550, Fisher Scientific, St. Louis, MO). Homogenates were centrifuged, and the supernatant drawn off. Protein concentration was determined by BCA (bicinchoninic acid) determination (Pierce, Rockford, IL). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [34]. Typically, 2-4 μg of protein were loaded per lane onto a 4% (w/v) SDS-polyacrylamide stacking gel with a 10% (w/v) SDS-polyacrylamide separating minigel. Electrophoresis was performed at 150 V for 30-45 min.

Proteins were transferred to Polyvinylidene difluoride (PVDF-Plus) membranes (Micron Separations Inc.) using a Trans-Blot Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA). To block remaining adsorption sites, membranes were incubated for 1 h at room temperature in Tris-buffered saline containing 3% (w/v) Carnation non-fat dry milk. Membranes were rinsed in NP-40 buffer and incubated overnight at room temperature with the desired antiserum. To remove nonspecifically bound antibodies, membranes were rinsed multiple times with NP-40 buffer. Membranes were then incubated for 2 h at room temperature in NP-40 buffer containing (^{125}I)-protein G (500,000 cpm/ml). To remove nonspecifically bound (^{125}I)-protein G, the membrane was rinsed multiple times in Sarkosyl buffer (pH 7.5), followed by a distilled H_2O rinse. The membrane was dried, wrapped in Saran Wrap and exposed to Kodak X-Omat AR film with a Cronex Lightening Plus intensifying screen at -70 °C.

Western blots were scanned by a Hewlett Packard Scanjet IIc using Deskscan software. Adobe Photoshop was used to crop, label, and size the images. An IBM compatible computer was used for all these procedures.

Phospholipase C activity assays: PLC activity assays were carried out by incubating a 0.1 ml volume of 50 mM Tris-Cl, pH 7.5, 10-7 M $CaCl_2$, 0.1 mg/ml BSA, 0.2 mM phosphatidylionositol (Sigma), 44,000 dpm phosphatidyl-[^{3}H]-inositol 4,5-bisphosphate (New England Nuclear), and *Drosophila* tissue extract for five min at room temperature essentially as described by [25]. Crude tissue extracts were prepared by grinding tissue in a buffer of 50 mM Tris-Cl, pH 7.5, 250 mM KCl, 0.05% sodium deoxycholate, 0.1 mM DTT, and 0.1 mM PMSF, using a teflon pestle in a 1.5 ml microfuge tube on ice. These homogenates were then centrifuged briefly at 12,000 x g to remove particulate matter. Protein concentration in crude homogenates were determined using the Bradford protein assay (Bio-Rad, Watford, Herts, England) with BSA as a standard and an appropriate amount of extract (amount empirically determined to yield linear results with respect to time) added to the reaction mixture. Reactions were stopped by precipitating in 5% trichloroacetic acid and quantifying emissions in the supernatant by liquid scintillation.

Immunocytochemistry: Procedures were outlined by Shim et al. [6] and detailed by Shim [35]. Heads were dissected and fixed in either 2.85% paraformaldehyde and 0.15% glutaraldehyde or 2.85% paraformaldehyde alone in 0.1 M Sorenson's phosphate buffered solution (pH 7.4) with 2% sucrose for 2.75 h. Fixed heads were washed several times in Sorenson's buffer. Dehydration with 30%, 50%, 70%, and 90% ethanol was followed by infiltration in L.R. White acrylic resin (London Resin Company/UK, Reading, Berkshire, England) overnight. Samples were embedded in the L.R. White in plastic gel capsules to avoid oxygen, then heat-polymerized at 55 °C.

Sections of approximately 1 μm were cut with glass knives (made with an LKB-7800 knife maker) using an Ultratome

III-type 8800 (LKB Instruments, Inc., Gaitherburg, MD) and fused to poly-L Lysine coated glass slides (Fisher Chem. Corp., Pittsburgh, PA). A barrier on the slide around samples was made with a hydrophobic slide marker (Research Products International Corp, Mount Prospect, IL). Samples were treated with sodium ethanolate for 45 min to etch the plastic, washed with ethanol and washed with deionized water. To minimize autofluorescence, samples were treated with 1% sodium borohydride, rinsed with deionized water and equilibrated in PBS (pH 7.4). Blocking was with PBS with 2% normal goat serum (Sigma) at room temperature for approximately 1 h.

Anti-PLC antibody was diluted at 1:200. To block nonspecific staining, primary antibodies were incubated with an equal amount of crude protein extracted from wild type fly heads after being diluted at room temperature with antibody in PBS containing 1% goat serum for 30 min to 1 h. FITC (fluorescein isothiocyanate) conjugated goat anti-rabbit IgG (Sigma) was diluted in PBS containing 1% goat serum at 1:50 ratio and applied. Samples were incubated at 37 °C for 1 h, rinsed in PBS, and rinsed in deionized water. The samples were then mounted in antifade mountant (2.5 g of DABCO [Sigma] dissolved in 10 ml of 10X PBS [pH 7.4] and 90 ml of glycerol) and coverslipped. The edge of the coverslip was sealed using clear finger nail polish (L'Oreal Super Top Coat) which was found to have very low fluorescence. Slides were kept in a -20 °C freezer.

Slides were viewed with a standard Leitz Dialux fluorescent microscope (Leica, Inc, Deerfield, IL). Standard fluorescent images were captured and printed using an Optronics (TEC-470, Goleta, CA) camera and Sony RGB video printer (UP-5200MD). Images were transferred to a PC computer with a Coreco Oculus TCX frame grabber (St. Laurent, Quebec). A confocal microscope, Bio-Rad MRC-600 fitted with a Kr/Ar laser on a Nikon Optiphot, was also used to obtain images. Excitation at 488 nm was supplied by fluorecein optics and images were acquired by Bio-Rad's COMOS software on an IBM-compatible host computer.

Image processing was performed using Image Pro Plus and Adobe Photoshop on the Windows operating system. Images were also analyzed using the freeware application NIH Image, version 1.61, running on the Macintosh OS. All the images were stored in optical disks by the use of a magneto-optical disk drive (APS technologies, Kansas City, MO).

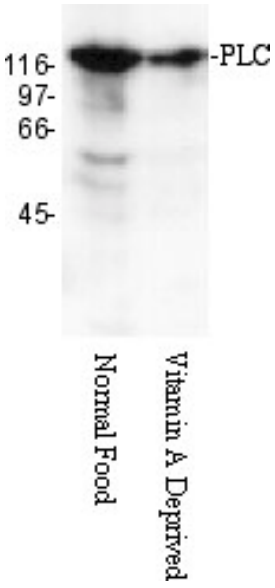

Figure 1. Western blot probed with anti-phospholipase C antibodies. The norpA gene encodes for PLC (130 kDa) and is necessary for proper phototransduction. PLC is greatly reduced in vitamin A deprived adults as compared to controls.

RESULTS

Figure 1 is a western blot that documents our study of the effects of retinoids upon *Drosophila* PLC. The PLC band (130 kDa) [25] is apparent in flies reared on a retinoid replete medium. Retinoid deprivation substantially decreased but did not eliminate the band. The stained bands at lower molecular weights are likely PLC degradative products. This experiment was replicated four times.

Activity analysis confirmed the western blot finding in that PLC activity was significantly reduced but not eliminated by deprivation relative to vitamin A replete flies reared on normal medium (Figure 2). For comparison, data from a positive control (w^{A35}) and a negative control ($norpA^{P24}$) are shown.

The immunocytochemical staining using anti PLC-antibodies confirmed the earlier demonstration [14] that PLC is localized to the R1-6 + R7 rhabdomeres (Figure 3A). Staining with a different antibody that does not label rhabdomeres demonstrated that rhabdomeres in white-eyed *Drosophila* have no detectable fluorescence of their own [6]. Rhabdomeres in flies reared on Sang's medium plus β-carotene (Figure 3B), looked like those of flies reared on normal cornmeal-β-carotene food (Figure 3A). Rhabdomeres seemed to fluoresce as brightly in deprived flies (Figure 3C) as in the flies grown on cornmeal-β-carotene food (Figure 3A) or β-carotene supplemented Sang's medium (Figure 3B). Rhabdomeres from flies reared

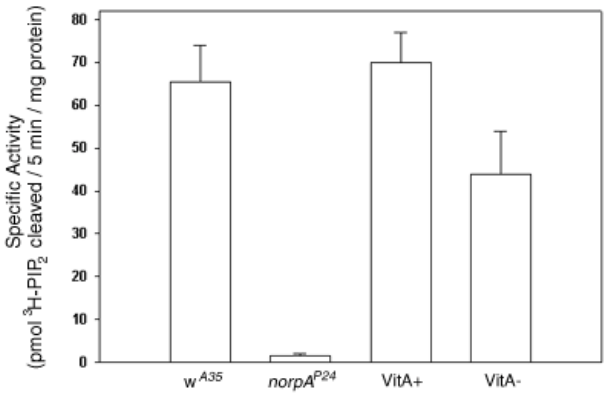

Figure 2. PLC activity analysis. PLC activity analysis comparing white-eyed (w^{A35}) controls with $norpA^{P24}$ mutants. Vitamin A deprivation decreases PLC activity relative to vitamin A replete controls.

on yeast-glucose food (Figure 3D), Sang's medium supplemented with beef brain-heart infusion (Figure 3E), and Sang's medium plus retinoic acid (Figure 3F) looked like rhabdomeres of retinoid deprived flies (Figure 3C) in that they showed equally bright PLC labeling. Recall that these latter chromophore deprivation manipulations (Figure 3D-F) had been considered useful since they eliminated opsin but not ninaE mRNA [3].

Figure 4A shows normally fluorescing rhabdomeres resulting from vitamin A replacement, with carrot juice given to deprived flies for 3 days. Replacement with β-carotene yielded a similar profile, specifically with large and bright rhabdomeres, whereas replacement with retinoic acid resulted in flies with small but bright rhabdomeres like those in the chromophore deprived flies (Figure 3C-F and data not shown). Figure 4B shows the lack of fluorescence in the norpA^{P24} mutant. In order to examine the relationship between functional opsin and PLC expression, some ninaE mutants were tested. Figure 4C,D show ninaE^{oI17} and ninaE^{ora} respectively. The

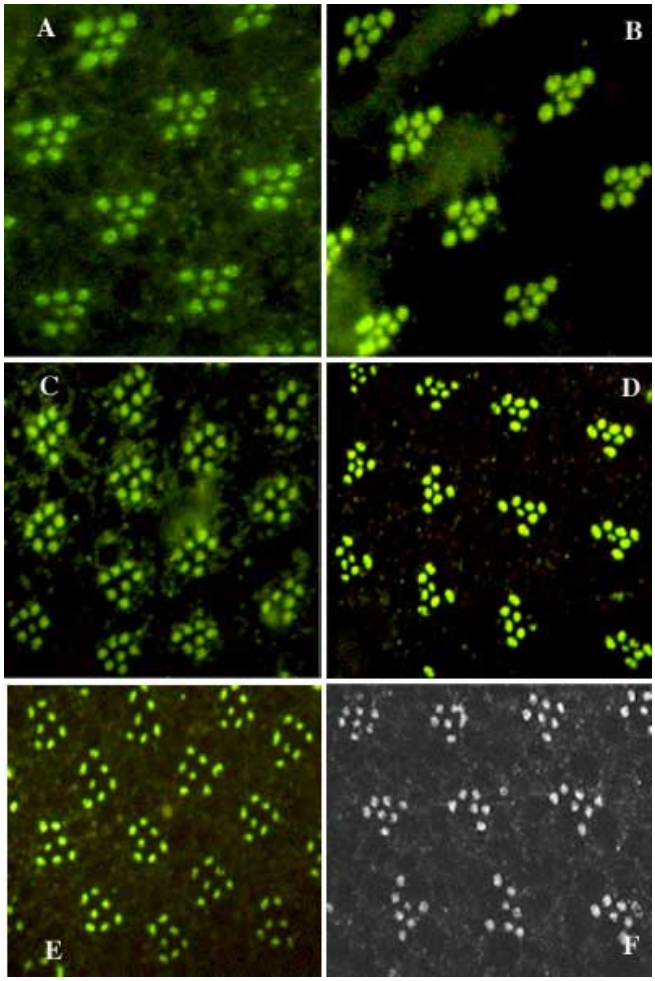

Figure 3. Immunocytochemistry for PLC. Immunocytochemistry for PLC in white-eyed, otherwise wild-type flies, retinoid replete, and retinoid or chromophore deprived. **A**: standard diet, bright fluorescence in R1-7 rhabdomeres, standard fluorescence micrograph. **B**: rearing on Sang's medium supplemented with β-carotene from egg to adult (one generation) showing the same bright fluorescence as for normal diet as expected, standard fluorescence micrograph. **C**: retinoid deprived; note that rhabdomeres fluoresce brightly but that the size of rhabdomeres may be decreased, standard fluorescence micrograph. **D**: rearing on yeast-glucose diet, showing same features as for deprived diet, standard fluorescence micrograph. **E**: rearing on Sang's medium supplemented with Beef Brain-Heart Infusion from egg to adult (one generation) showing the same features as for retinoid deprived diet, standard fluorescence micrograph. **F**: rearing with retinoic acid supplemented Sang's medium for one generation showing same features as for deprived diet, confocal micrograph.

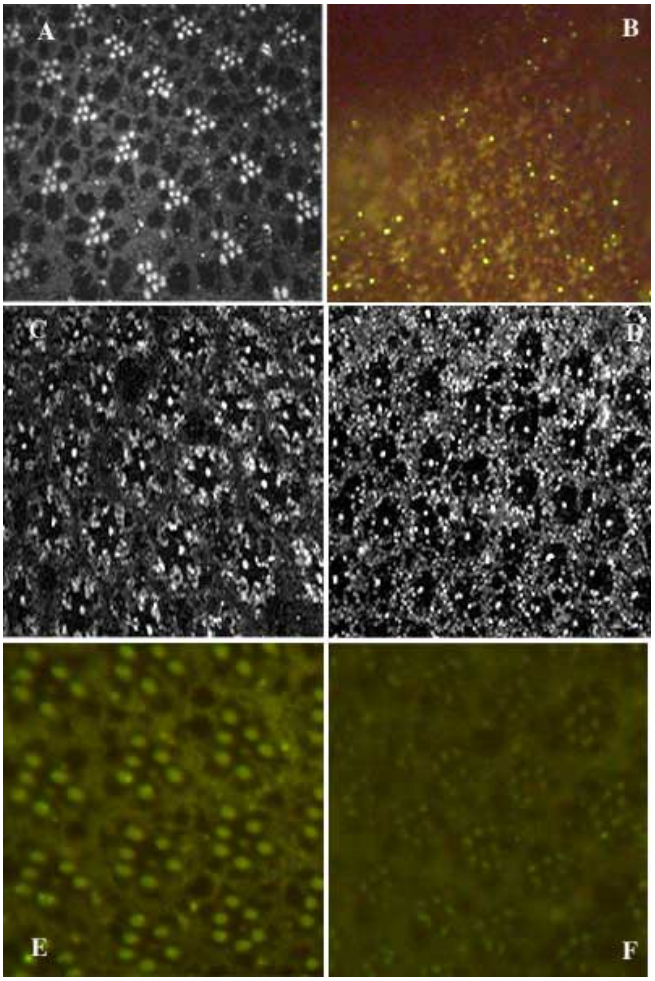

Figure 4. Fluorescence micrographs with PLC antibody. Fluorescence micrographs prepared with PLC antibody, repletion manipulations, and selected mutants. **A**: 3 days carrot juice replacement of deprived flies, showing the recovery of rhabdomere size, confocal micrograph. **B**: negative control, the mutant white-eyed norpA^{P24}, standard diet, standard fluorescence micrograph, no rhabdomere fluorescence detected as expected. **C**: the white-eyed mutant ninaE^{oI17}, standard diet, confocal micrograph; note normal level of PLC detected in R7 rhabdomeres. **D**: the white-eyed mutant ninaE^{ora}, standard diet, confocal micrograph; note normal level of PLC detected in R7. **E**: the transformant TI-6 rescued from white-eyed norpA^{P24} by norpA minigene driven by ninaE promoter, showing PLC detected in only R1-6 rhabdomeres, standard diet, standard fluorescence micrograph. **F**: the transformant TI-6 rescued from white-eyed norpA^{P24} by norpA minigene driven by ninaE promoter, showing no fluorescence emission, retinoid deprived diet, standard fluorescence micrograph.

ninaE^{ol17} and ninaE^{ora} mutants express PLC in R7 and R8 rhabdomeres. Additionally, there is fluorescence outside the center of the rhabdomere. This may or may not be PLC-based and may be related to the finding that newly-emerged ninaE^{ora} flies do have R1-6 rhabdomeres that diminish with age [31].

Vitamin A manipulations were extended to the transgenic flies rescued by a chimeric norpA minigene driven by the ninaE gene promoter. The TI-6 allele [14] was reared on either regular or deprivational medium. Since the ninaE promoter drives expression into R1-6, PLC labeling is only unequivocal in R1-6 rhabdomeres in retinoid-replete flies (Figure 4E); R7/8 fluorescence is at about the level of background (non-rhabdomeric tissue). Vitamin A deprivation (Figure 4F) eliminates the PLC expression seen in vitamin A-replete TI-6. This result is a critical piece of evidence that retinoids control transcription at the level of the opsin gene promoter since PLC, unlike rhodopsin, does not utilize retinoids translationally.

DISCUSSION

Since retinoids control transcription of opsin and RFABG genes, it was crucial to test whether other phototransduction molecules might be regulated by retinoids. Our results support the alternative, that rhodopsin reduction causes secondary decreases in the transduction machinery. The PLC activity and the PLC band were reduced by retinoid deprivation. PLC is the second molecule downstream from rhodopsin in the phototransduction cascade. Retinoid deprivation reduces PLC activity to 63% of that of the vitamin A replete control. Furthermore, the vitamin A replete control has an activity very close to another control (w^{A35}) in an experiment that verifies the expected zero activity in norpA^{P24} mutants. Although it was not quantified, the western blot also suggests that vitamin A deprivation decreases PLC to 1/2 or 1/3. In summary, activity analyses and western blots are consistent with deprived flies having smaller rhabdomeres [15].

Immunocytochemistry reveals that retinoid deprivation does not eliminate PLC expression in rhabdomeres. Deprived flies (Figure 3C) seemed to have rhabdomeres as brightly labeled as those of flies reared on retinoid replete foods (Figure 3A,B). Deprived flies (Figure 3C) were just like flies deprived of chromophore precursors but not deprived of other activators of opsin and RFABG expression (Figure 3D-F).

The impression from this study's immunocytochemistry is that rhabdomeres from retinoid and chromophore deprived flies are reduced in size (compare Figure 3C-F with retinoid replete controls, Figure 3A,B). However the present study is not optimum to prove this point due to the low magnification of this light microscopy and the fact that rhabdomeres taper in the distal to proximal direction; the plane of section could not be controlled. However, thorough morphometric comparisons from high magnification electron micrographs where the plane of section from which measurements were taken was controlled showed that deprived flies have smaller rhabdomeres than replete and carrot juice replaced flies [15].

It seems likely that the reduction of PLC, as detected in activity analyses and western blots, is secondary to lack of visual pigment. PLC was also found in other parts of the *Drosophila* body, including thorax, abdomen, and legs [25]. This suggests that PLC may be involved in signaling pathways not associated with vision. If retinoids exert their effects on PLC by regulating opsin, it seems unlikely that retinoid deprivation would reduce PLC levels in these other parts of the body, and this hypothesis could be tested.

Vitamin A manipulations on the the transgenic flies (TI-6) showed that, when the norpA coding sequence is driven by the ninaE promoter, the gene product is affected by retinoid deprivation. This is one of the strongest pieces of evidence substantiating the earlier finding [3-5] that retinoids control ninaE transcription at the promoter level. By contrast, the results of this study suggest that retinoids do not directly affect the transcription of the PLC gene at the level of the PLC gene's promoter. This finding is consistent with the differences in the norpA promoter from those of many other receptor-specific genes [36].

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