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## Light activation of phospholipase A<sub>2</sub> in the photoreceptor of the crayfish (*Procambarus clarkii*)

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**Abstract.** Retinal lipids of crayfish, kept at 4°C under continuous darkness for 3 weeks, consisted mainly of phosphatidylcholine (PC) and phosphatidylethanolamine (PE); sphingomyelin (SM), phosphatidylinositol (PI) and phosphatidylserine (PS) were minor contributors. PI, involved in the phototransduction cascade, never reached greater concentrations than 7% of the total. High concentrations of polyunsaturated fatty acids (PUFA) such as 20:4n-6, 20:5n-3 and 22:6n-3 (DHA, docosahexaenoic acid) were present in PC, PE and PS, but scarce in SM and PI. In retinae of crayfish kept at 4°C in darkness for 3 weeks and then exposed to white light (6 h; ca. 4,500 lx), SM and PS remained seemingly unaffected. PC, however, significantly decreased within 10 min to 65% of the initial value and 50% at 180 min. To study the reduction of PC, lipids of retinae suspended in physiological solution with/without phospholipase C (PLC) and phospholipase A<sub>2</sub> (PLA<sub>2</sub>) inhibitors such as DMDA (=DEDA), manoalide, ET-18-OCH<sub>3</sub>, and U-73122 were measured. Only free fatty acids (FFA) of retinae with inhibitors of PLA<sub>2</sub> like DMDA and manoalide decreased. Retinae irradiated by white light for 3 h displayed a significant reduction of PC, compared with those that had remained in continuous darkness. However, the PC of retinae with PLA<sub>2</sub>-inhibitors was not decreased by light. Our results provide evidence that not only photoreceptor cell PLC, but also PLA<sub>2</sub> is activated by light.

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**Key words:** compound eye, crayfish, fatty acid composition, phospholipid, phospholipase A<sub>2</sub>, phototransduction, vision

## INTRODUCTION

The very recently suggested concept (Kiselyov and Muallem 1999) based on evidence provided by Chyb et al. (1999) that cation-selective channels in the visual membranes of the compound eye may be regulated by polyunsaturated fatty acids (PUFA), casts a new light on the old question of light-induced photoreceptor damage in the crustacean eye (Meyer-Rochow 1994). Photoreceptor membranes are the site of photoexcitation and according to the model of Singer and Nicholson (1972) the visual pigments float as trans-membrane molecules in the lipid bilayer, the latter being composed of the fatty acid chains of the contributing phospholipids (Blasie and Worthington 1969).

Proper functioning of any cell membrane is dependent on its fluidal stability, which, in turn, depends very significantly on the composition of its membrane lipids. We were able to show that the amounts of unsaturated and saturated fatty acids (UFA and SFA) apparently determined to a large extent the homeoviscous properties of crayfish photoreceptor membranes under thermal and photic stress (Kashiwagi et al. 1997). If, however, fatty acids are also involved in cation channel control, photoreceptor damage following exposure to light could be caused by PUFA-mediated changes in receptor cell  $Ca^{2+}$ -concentrations, independent from the effects of PUFA on membrane fluidity (Meyer-Rochow 2000).

To test this hypothesis, it was necessary to explore quantitatively the changes in the various phospholipid amounts and the activities of some relevant phospholipase (or lipases) during and after illumination to light. If the production of PUFA was facilitated by the action of phospholipase  $A_2$  (PLA<sub>2</sub>), or diacylglycerol (DAG) lipase on DAG, then, as also suggested by Kiselyov and Muallem (1999), an inhibition of PLA<sub>2</sub> or DAG lipase, or both, should block phototransduction. A blocked phototransduction could then exert an influence on some of the known light-induced changes like, for instance, the reduction of phosphatidylcholine (PC) levels. The roles of phosphatidylinositol (PI) as a component of the rhabdom membrane and phospholipase C (PLC) as a step in the phototransduction cascade have already been established (Smith et al. 1991). However, the functions of the other photoreceptor cell phospholipases during an exposure to light have remained largely unknown.

We tackled this problem by investigating lipid compositions and fatty acids of firstly isolated dark adapted retinæ and, secondly, of retinæ suspended in physio-

logical saline with/without inhibitors of lipid metabolism such as DMDA, (= 7,7-diamethyl-5,8-eicosadienoic acid: DEDA), manoalide, ET-18-OCH<sub>3</sub>, and U-73122 before and after an exposure to light. The goals of this study were to check whether PUFA-production was indeed mediated by the action of PLA<sub>2</sub> on membrane lipids and whether the application of PLA<sub>2</sub> inhibitors would reveal the degree of PLA<sub>2</sub>-involvement in membrane stability.

## METHODS

### Animals

Crayfish (*Procambarus clarkii*: Astacura, Decapoda) were obtained from a commercial supplier in Yokohama and maintained in a low-temperature (4°C) incubator (Eyela LTI-600ED, Tokyo-Rikakikai, Tokyo) under continuous darkness for 3 weeks. Water temperature was  $4 \pm 1^\circ\text{C}$  and the water level in the aquaria was 1.2-1.5 cm. For each experimental analysis six eyes of 3 crayfish were used.

### Light exposure of tissue

To obtain samples of the retinal tissue of the compound eyes of crayfish, the retinæ of the animals were isolated from the compound eyes by carefully freeing them from their corresponding dioptric structures such as corneae and crystalline cones. The retinæ were then collectively incubated for 6 h at 4°C in physiological saline with/without the metabolic inhibitors of the phospholipids. Exposure to white bright light for 3 h at the same temperature followed. The light source was a common fluorescent white lamp (FL20SS-EX-D of the daylight type, Matsushita, Tokyo). Light intensity measured at the surface of the physiological saline was approximately 4,000-5,000 lx.

### Inhibition experiments

Metabolic inhibitors of phospholipids, used in these experiments as PLC-inhibitors, were ET-18-OCH<sub>3</sub> (1-O-Octadecyl-2-O-methyl-sn-glycero-3-phosphorylcholine, BIOMOL research Laboratories, Inc., USA: Herrmann and Neumann 1986, Powis et al. 1992) and U-73122 (1-(6-((17s-3-methoxyestra-1,3,5(10)-trien-17-yl)-amino)hexyl)-1h-pyrrole-2,5-dione, BIOMOL research Laboratories, Inc., USA: Smith et al. 1990, Thompson et

al. 1991); as PLA<sub>2</sub> inhibitors the following substances were used : DMDA (7,7-dimethyl-5,8-Eicosadienoic Acid, Cayman chemical, USA: Lister et al. 1989) and manoalide (4-[3,6-Dihydro-6-hydroxy-5-[4-methyl-6-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-hexenyl]-2H-pyran-2-yl]-5-hydroxy-2(5H)-furanone, RBI Research Biochemicals International, USA: Mayer et al. 1987).

To prepare the inhibitor stock solutions, ET-18-OCH<sub>3</sub>, U-73122, and manoalide were dissolved in DMSO while DMDA was dissolved in ethanol. Final concentration of each inhibitor used in the physiological saline was 1.72 µM (U-73122), 10 µM (ET-18-OCH<sub>3</sub>), 10 µM (DMDA), and 12 µM (manoalide). As for the controls of the inhibition experiments, the same concentration of DMSO (0.2% by volume) but without any inhibitor in the physiological saline was used.

#### **Preparation of tissue for analysis by two dimensional thin-layer and gas chromatography**

In order to obtain phospholipids and fatty acid components from the animal eyes, total lipids were extracted from specimens by using the modified method of Folch et al. (1956). Small pieces of tissue were homogenized for about 3 min in excess volumes (2 ml) of chloroform / methanol (2:1, by vol.) by Polytron PT3000 (Kinematica, Luzern) with type 3012/2EC blade. The extract was separated and the residue was re-extracted with CM. The combined extracts were evaporated under reduced pressure below 35°C. The total lipids thus obtained were dissolved in 4 ml of CM and non-lipid substances were removed by partition through the addition of 1 ml of water. The lower organic phase was washed again with 2 ml of methanol / water (1:1 by vol.). The total lipids thus obtained were dried up under a stream of nitrogen and kept sealed in 1 ml vials (Wheaton, Millville, NJ). Oxidation was prevented by keeping the samples at -35°C under the nitrogen whenever possible.

#### **Analysis of phospholipids by two dimensional thin-layer chromatography**

Two dimensional thin-layer chromatography was carried out on a 10 (10 cm HPTLC-plate (Merck, Darmstadt), by using a solvent system of chloroform / methanol / 28% ammonia (6:4:1 by vol.) for the first, vertical dimension and chloroform / acetone / methanol / acetic acid / water (50:20:15:10:6 by vol.) for the second, horizontal dimension. For the identification of in-

dividual lipids, their RF values were compared with those of authentic standards. The spots were also detected by various reagents for their specific identification: Dittmer reagent (Dittmer and Lester 1964) for the organic phosphorous compound, ninhydrin reagent (Lea et al. 1955) for amino residues, Dragendorf reagent (Beiss 1964) for choline, and Molish reagent (Siakotos and Rouser 1965) for carbohydrates. For the identification of alkaline-stable lipids, portions of the total lipids were treated with 0.5 N-methanolic NaOH prior to the chromatography.

For the separation of lipids developed on the TLC plate, the spots detected by spraying 0.01% (wt/vol.) primuline in acetone:water (4:1 by vol./vol.) were marked with pencil (Ostrander et al. 1988). Silica gel scratched from each mark was suspended in chloroform / methanol / water (2:1:0.1 by vol.) by using a vortex mixer followed by immersion in a sonicator bath for several minutes. The suspension was layered over a small column of Iatrobeads 6RS-8060 (Iatron, Tokyo) packed 1-2 cm in height in a Pasteur pipette and eluted by at least 10 column volumes of the same solvent. The eluent was evaporated to dryness under the stream of nitrogen. Fatty acid components of the eluted lipids were derivatised by methanolysis with 3% methanolic HCL for 16 h (Sweely and Walker 1964). Fatty acid methyl esters in the methanolysate were extracted by hexane for the gas-liquid chromatographic analysis. The residual methanolic phase was again evaporated to dryness under a stream of nitrogen for the analysis of its phosphorous content by the method of Bartlett (1964) with slight modifications by using 0.01% aminonaphthol sulphonic acid as the reducing agent.

#### **Analysis of fatty acids by gas chromatography**

A split-splitless gas chromatograph apparatus of Shimadzu (GC-14A), equipped with a capillary column of type DB-225 (J and W. Scientific, 30 m (0.25 mm I.D., 0.15 µm thickness) was used with a temperature programming initially from 60°C to 180°C at 30°C/min and after leaving for 2 min up to 240°C at 2°C/min. The data processor was a Shimadzu Chromatopak C-R6A. The relative amounts of each fatty acid methyl ester were calculated from the integrated areas of the corresponding peaks through comparisons with those of the reference sample. Tests of statistical significance (Welch's *t*-test) were carried out for the cases referred to in the text (significant =  $P < 0.05$ ; highly significant =  $P < 0.01$ ) and the

nomenclature used for the fatty acids is that given in Eguchi et al. (1994).

## RESULTS

### Lipid compositions of the compound eye retina

Lipid compositions of crayfish compound eye retinæ kept at 4°C under continuous darkness for about 3 weeks were analysed by two dimensional thin-layer chromatography. The detected components of lipid in the compound eye were free fatty acids (FFA), sphingomyelin (SM), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) (Fig. 1). PC and PE dominated, while SM, PI, and PS never reached concentrations greater than 15% of the total and can be considered minor components.

### Fatty acid composition of each phospholipid

The fatty acids of each phospholipid of the crayfish retinæ kept at 4°C under continuous darkness for 3 weeks

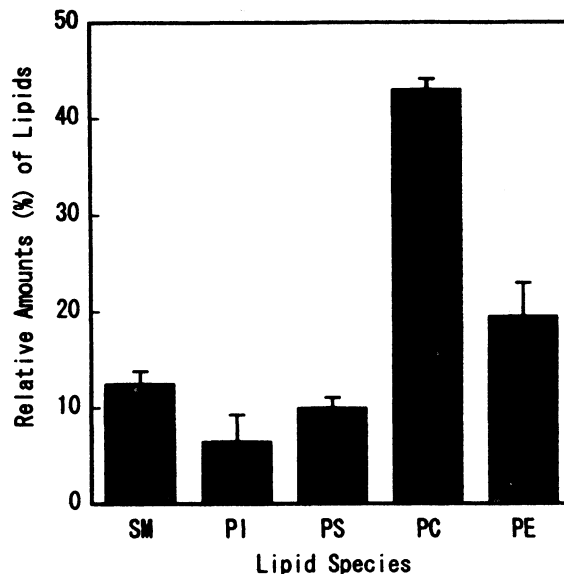


Fig. 1. Lipid compositions of retinæ from crayfish kept under continuous darkness for about 3 weeks at 4°C. SM, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

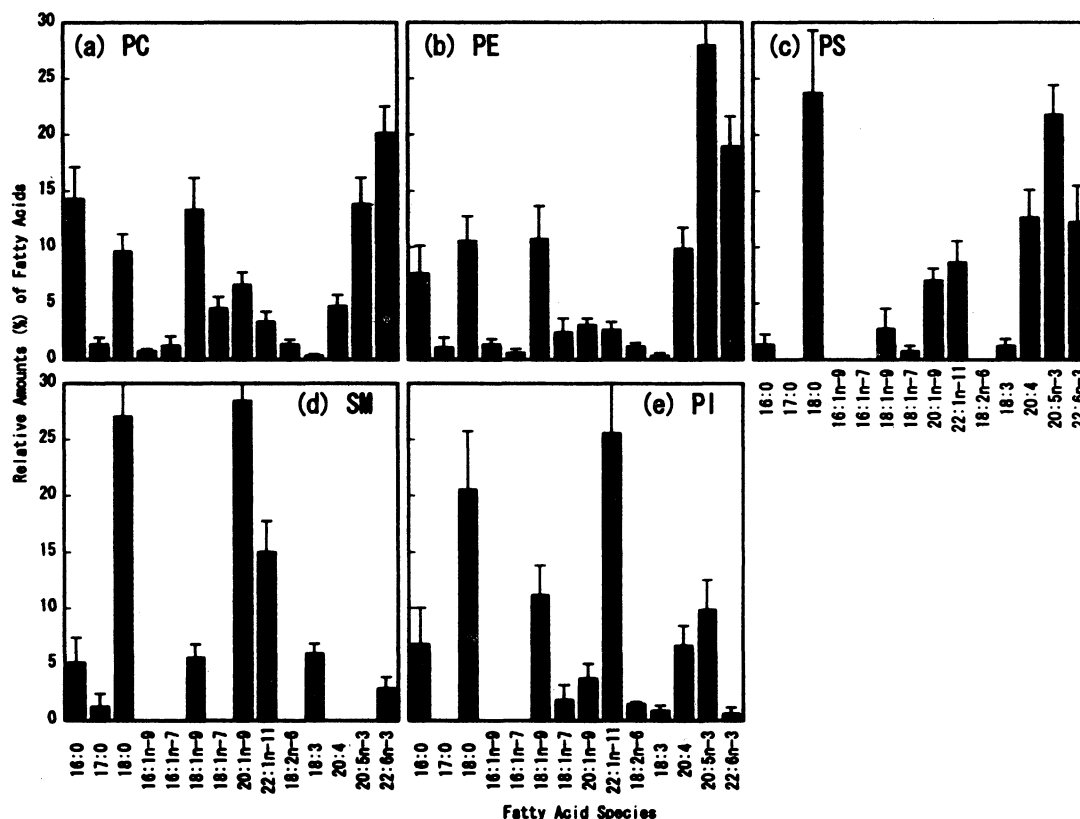


Fig. 2. Fatty acid composition of phospholipids from retinæ of crayfish kept under continuous darkness at 4°C. a, phosphatidylcholine; b, phosphatidylethanolamine; c, phosphatidylserine; d, sphingomyelin; e, phosphatidylinositol.

were analysed by gas-chromatography. Generally, of all the species of fatty acids contained in each phospholipid, 16:0 and 18:0 dominated amongst the SFA, while 18:1n-9, 20:1n-9, and 22:1n-11 represented the main UFA. Additionally, PUFAs such as 20:4n-6, 20:5n-3, and 22:6n-3 (docosahexaenoic acid, DHA) were present in significantly large amounts in PC, PE, and PS, respectively (Fig. 2a, b, and c). However, such PUFAs were minor components in SM and PI (Fig. 2d and e).

**Quantitative changes in phospholipid composition following exposure to white light**

Isolated retinæ of crayfish kept at 4°C under continuous darkness for 6 h in physiological saline without inhibitors of phospholipids were exposed to white light (4,000-5,000 lx) for 6 h. The quantitative changes in the phospholipid compositions of the retinæ were measured by two dimensional thin-layer chromatography at 10, 60, 120, 180, and 360 min from the onset of the light exposure. Relative amounts (%) of phospholipid species per total detected phospholipids were also calculated. Both SM and PS remained seemingly unaffected by the exposure to light. However, the amount of PC showed a significant decrease to less than 65% of the initial value within 10 min ( $P<0.01$ ), a further decrease to 50% at 180

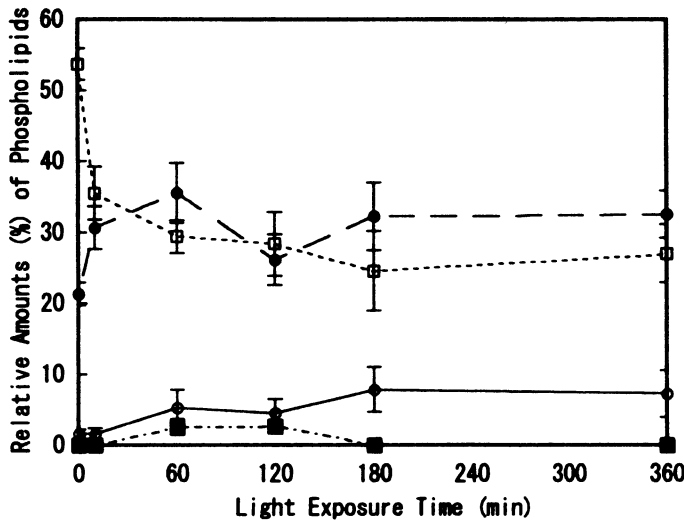


Fig. 3. Relative changes in the amounts of four retinal phospholipids (LP, PI, PE, and PC), caused by an exposure to light (4,000-5,000 lx) in crayfish that had previously been kept under continuous darkness at 4°C. Filled squares, lyso-phospholipid; filled circles, phosphatidylethanolamine; open circles, phosphatidylinositol; open squares, phosphatidylcholine.

min and a stabilization at that level thereafter (Fig. 3). The amount of PE was increased relative to the reduction of PC by the exposure to light ( $P<0.01$ ). PI gradually increased up to 7% ( $P<0.01$ ) and small amounts of lyso-phospholipid (LP) appeared at 60 and 120 min.

**Effects of inhibition of lipid metabolic enzyme**

As explained in detail under Methods retinæ of crayfish kept at 4°C under continuous darkness for about 3 weeks were carefully separated from their corresponding dioptric structures such as corneae and crystalline cones and collectively suspended in physiological experimental solution with and without lipid metabolic inhibitors such as DMDA, manoalide, ET-18-OCH<sub>3</sub>, and U-73122 for 6 hours.

**Relative amounts of free fatty acids (FFA)**

Compared with the retinæ of crayfish kept at 4°C under continuous darkness for 3 weeks, those kept at the same temperature, but exposed to white light (4,000-5,000 lx) for 3 h, displayed significantly higher levels ( $P<0.01$ ) of FFA (Fig. 4). However, the amounts of FFAs of retinæ kept in physiological saline to which inhibitors of PLA<sub>2</sub> such as DMDA and manoalide were added decreased significantly ( $P<0.01$ ). Relative amounts of FFA were not statistically significantly changed by ET-18-

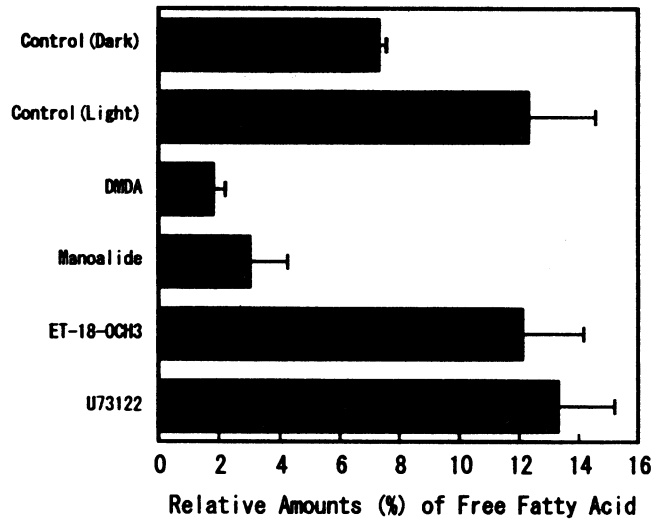


Fig. 4. Relative amounts of free fatty acids of retinæ suspended in physiological saline with and without inhibitors of lipid metabolism and exposed to bright white light (4,000-5,000 lx).

OCH<sub>3</sub> and U-73122 employed as PLC inhibitors. These results suggest that both DMDA and manoalide as PLA<sub>2</sub>-inhibitors affect the lipid metabolism in the most important way.

### Relative amounts of phospholipids

Compared with the retinae of crayfish kept at 4°C under continuous darkness for 3 weeks, those kept at the same temperature, but exposed to white light (4,000-5,000 lx) for 3 h displayed significantly lower levels ( $P < 0.01$ ) of PC (Fig. 5). However, the PCs of retinae kept in the physiological saline to which inhibitors of PLA<sub>2</sub> such as DMDA and manoalide were added did not decrease as a consequence of the exposure to light. The PCs of retinae kept in physiological saline that included ET-18-OCH<sub>3</sub> or U-73122 as PLC inhibitors showed decreases not unlike those observed in the controls exposed to light. The amounts of PE, PI, and PS, following the addition of the inhibitors of PLC and PLA<sub>2</sub>, did not show significant differences compared with those of the control when exposed to light.

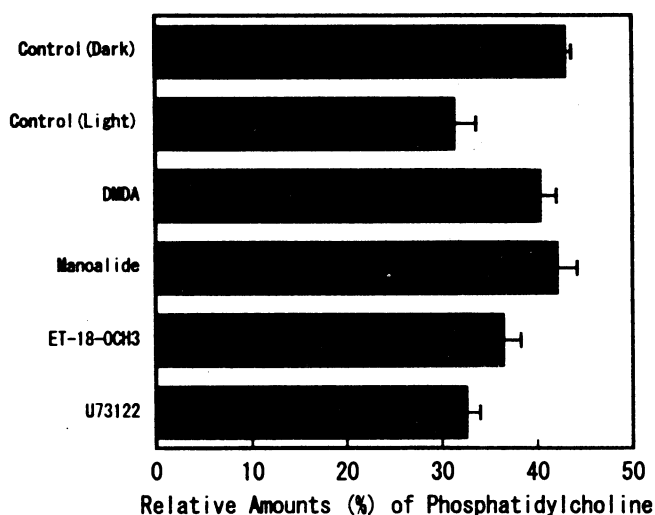


Fig. 5. Relative amounts of phosphatidylcholine of retinae suspended in physiological saline with and without inhibitors of lipid metabolism and exposed to bright white light (4,000-5,000 lx).

## DISCUSSION

The lipids of biological membranes, generally, such as endoplasmic reticulum, mitochondrial membranes, plasma membranes, lysosomal membranes, and nuclear

membranes are composed of PC, PE, SM, PI, and PS (Van Meer 1989). Both PE and PC are dominating in the retinae of crayfish (Meyer-Rochow et al. 1999) and Fig. 1 of this paper confirms that earlier result. This fact indicates that PC and PE are important components of the retinal phospholipids in crayfish, and are probably involved in the stability of the highly specialized biological membrane type represented by the photoreceptor rhabdoms. High concentrations of PC are reached in the retinae of animals kept at 4°C in constant darkness (Fig. 1). When the retinae of the crayfish kept at 4°C under a light/dark cycle of 12/12 hours (L/D:12/12) were examined, the amounts of PC were lower than those of retinae from individuals kept under constant darkness for 3 weeks (data not shown). Such observations suggested that the disintegrating metabolism of PC in the retina of crayfish was activated through stimulation by light, but did not reveal much about the mechanism of that phenomenon.

It has been documented that food (Leger et al. 1986), season (Littlepage 1964, Bennett et al. 1997) and environmental light (Kashiwagi et al. 1997) can affect unsaturated fatty acid concentrations in crustaceans. Levels of DHA, in particular, were found to drop following a prolonged exposure to light (Kashiwagi et al. 1997). Since both PC and PE contained large amounts of DHA (about 20%, Fig. 2) it would seem fair to assume that the reduction of DHA following an exposure to light can be attributed to a loss of DHA mainly from PC and PE. Since the bulk of the retina in the crayfish eye is made up of the rhabdoms visual membranes, i.e. the microvilli, all our conclusions relate primarily to the latter and much less to the non-microvillous membrane components of the retinal cells.

As described in the results, the amount of PC decreased markedly, while FFAs increased, following the illumination by bright light. The present experiments revealed that during the exposure to light of the retinae immersed in the physiological solutions containing the PLA<sub>2</sub>-inhibitors DMDA and manoalide, the amounts of FFA did not increase (Fig. 4), while the amounts of PC did not decrease (Fig. 5). This finding indicates that DMDA and manoalide effectively inhibited the disintegrating metabolism of PC, usually responsible for the production of FFAs. In other words, in normal crayfish retinae the increases of FFAs and the decreases of PCs appear to be due to an activation by light of PLA<sub>2</sub>.

Of the various phospholipid species monitored, only PC decreased significantly in response to illumination,

suggesting that PLA<sub>2</sub> is specifically functional, or effective, in connection with PC. Phospholipid, generally, is degraded into two components, FFA and lyso-phospholipid (LP) by PLA<sub>2</sub> (Bell and Coleman 1980, Dawidowicz 1987, Bishop and Bell 1988, Van Meer 1989). In the present experiments, along with the increases of FFAs as mentioned above, the expected increase of LP under light illumination was also confirmed (Fig. 3). An activation of PLA<sub>2</sub> through light was originally reported from rod outer segments of the bovine retina by Jelsema (1987), who suggested that the activation of PLA<sub>2</sub> could accompany a transducin-dependent mechanism. For the invertebrate compound eye, however, and in the light of the suggested role of PUFAs in cation channel control (Chyb et al. 1999, Kieslyov and Muallem 1999), our demonstration of the effects of PLA<sub>2</sub>-inhibitors is new and of considerable significance.

Regarding the established role of PI in the phototransduction cascade of the compound eye photoreceptor cell (Smith et al. 1991), our present experiments show that even during the exposure to light, the amount of PI remains almost unchanged from the pre-exposure level. This observation indicates that some synthesis of PI must occur simultaneously during the phototransduction-related PI-loss that is thought to occur as a response to the light exposure. In other words, synthetic metabolism of PI as well as PLA<sub>2</sub> activity both appear to be jointly dependent on light illumination.

Finally, our results provide evidence that not only PLC, a known component of the phototransduction cascade, but also PLA<sub>2</sub> is activated in photoreceptor cells through light. The precise physiological meaning of the documented PLA<sub>2</sub> activation in crayfish photoreceptors by light illumination remains to be revealed through future investigations. However, one might expect PLA<sub>2</sub> to play a role not only in regulating PUFAs (and thus cation channels), but also to be involved in protecting visual membranes against the damage caused by peroxidized fatty acids (Hölzel and Spiteller 1995), in this case involving predominantly DHA from PC.

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