Effects of Chloroquine on the Photosensory Membrane Turnover and the Ultrastructure of Lysosome-Related Bodies of the Crayfish Photoreceptor

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The effect of chloroquine in combination with bright light on the ultrastructure of crayfish (*Orconectes limosus* R.) photoreceptors was investigated *in vivo*. Chloroquine had several effects upon the crayfish retina. Multivesicular bodies (MVB) that are involved in lysosomal degradation of photosensory membrane were altered in ultrastructure. MVB frequently contained smaller MVB in a state of more advanced membrane degradation. Additionally screening pigment-like granules appeared in MVB. MVB accumulated in and filled the retinular cell cytoplasm just proximal or distal to the basement membrane which indicated inhibition of photosensory membrane degradation. Under chloroquine treatment rhabdom degradation appeared to be inhibited, as rhabdom diameter was less reduced under these conditions.

Also chloroquine caused accumulation of screening pigment granules in glial cells within the lamina ganglionaris.

Introduction

Light-induced photosensory membrane breakdown is a general phenomenon of photoreceptors of invertebrates. However, the degree of this effect differs between species or phyla. Whereas no changes in size of rhabdomeres after light exposure were reported for cuttlefish [1], rhabdomeric microvilli were found to be longer in the dark in insects [2, 3]. Dramatic rhabdom breakdown was observed in crabs [4], flies [5] and *Limulus* [6]. In crayfish, however, no large differences in rhabdom size occurred during a 12 h light and 12 h dark rhythm [7–9]. In crayfish photoreceptors, vesicles from broken down rhabdomeres accumulated in multivesicular bodies (MVB) [7, 10–12]. These MVB were degraded by lysosomal enzymes to organelles containing vesicles and whorls of membranes (combination or mixed bodies), or to organelles containing membraneous structures, the so-called lamellar bodies. MVB and lysosomal enzymes are generally involved in photosensory membrane degradation of invertebrates [1, 5, 12–18]. Also in crayfish, a large number of MVB can be observed in the retinular cell cytoplasm [7]. Nothing is known about the fate of these organelles.

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The origin of another organelle, the screening pigment granule, that also occurs in large numbers in photoreceptors of invertebrates and regulates light fluxes to the phototransductive membranes (see [19]) is unknown too. Although electron microscopic descriptions of the development of screening pigment granules in insects have been done [20–22] these processes are not well understood. Meyer-Rochow and Eguchi [23] propose that in crayfish screening pigment granules are formed from broken down microvilli, while Johnson and Gordon [24] describe a unique pathway that utilizes endoplasmic reticulum for screening pigment granule formation in the retina of an Opilionid (Arachnida). Screening pigment granules of invertebrates were found to consist of ommochromes which are synthesized from tryptophan [25, 26]. Recently, small screening pigment-like granules were found in the lamellar bodies and MVB of the crayfish retina after *in vitro* incubation with chloroquine [27] which is an inhibitor of lysosomal enzyme activity [28].

The present study was done to investigate whether chloroquine inhibits photosensory membrane degradation of crayfish.

Materials and Methods

Light- and electron microscopy

Eyestalks from four adult crayfish (*Orconectes limosus* R.) kept in outdoor aquaria under a natu-

ral light cycle at 15–18 °C were removed at midday (light-adapted). Another two groups of crayfish were kept at 15 °C vor 5 days under constant illumination (9.2 mW/cm^2) measured at the water surface. These animals were also sacrified at midday. The water depth in the aquaria was 20 cm. The first group of 3 animals was only subjected to light whereas in the second group three animals were additionally treated with 0.5 mg/day chloroquine (Sigma, Dreieich, Germany) for five subsequent days to inhibit lysosomal enzyme activity. The dosage of chloroquine was about 20-fold compared to that used for prophylaxis of malaria in children [29]. The drug was dissolved in 100 ml water and was transfused into the stomachs of the animals with the aid of a tube connected to a syringe. The animals showed normal behaviour and could be fed immediately after injection. The size of the animals varied only little and the body weights ranged from 22–25 g. After removing the eyestalks retinae were excised and fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer of pH 7.6 containing 2% sucrose and 2 mM $CaCl_2$ for several hours at 4 °C. Retinae were postfixed with 1% OsO_4 at room temperature in 0.1 M cacodylate buffer for 3 h, bloc-stained in 5% uranyl acetate in 70% alcohol for 1 h and embedded in Spurr's medium following standard techniques. Semithin sections (1 µm) were examined under a Leitz Orthoplan light microscope after staining with toluidine blue. Rhabdom diameter measurements were made from semithin sections oriented in the longitudinal plane according to [7]. Measurements were only made of rhabdom profiles which showed continuity with the 8th retinular cell at the distal end of the photoreceptor layer and whose profile extended down at least within 50 µm distally to the basement membrane. Measurement of the maximum diameter in these selected profiles provided a consistent sample point along the spindle shaped rhabdom. Ultrathin sections were stained with uranyl acetate and lead citrate and observed under a Philips EM 300.

Statistical methods

Student's t-tests were performed for measurements of rhabdom diameter.

Results

Light microscopic determination of rhabdom diameters from crayfish maintained under natural light cycle, permanent bright light and permanent bright light and additional chloroquine treatment

The mean value of rhabdom diameter and rhabdom length from the individual experimental animal groups are shown in Table I. Light micrographs of rhabdoms after prolonged illumination are shown elsewhere [27]. Differences of rhabdom width between chloroquine-treated crayfish and those that were not were significant ($p < 0.001$).

Electron microscopic observation of the crayfish retina after bright light exposure and additional chloroquine treatment

In the following section only alterations will be mentioned that were observed exclusively or, as in the case of glial cells containing pigment granules, more frequently after chloroquine treatment. Adjacent to the microvilli of each rhabdom (topographic position 1, Fig. 1) small screening pigment granules regularly revealed electron dense cores surrounded by an electron opaque material (Fig. 2, 3). Other granules revealed homogeneous electron opaque content (Fig. 2). The electron transparent space between limiting membrane and screening pigment granules occasionally contained vesicles (40–80 nm in diameter) that were identical

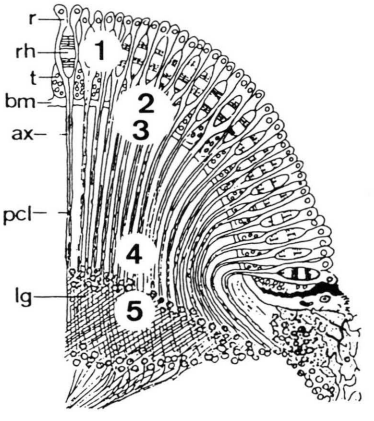


Fig. 1. Schematic drawing of a longitudinal section through an isolated crayfish retina. The numbered areas mark the areas of which photographs were taken (r = retinular cell, rh = rhabdom, t = tapetum cell, bm = basement membrane, ax = retinular cell axon, pcl = cluster of pigment granules, lg = lamina ganglionaris).

Plate 1. Effect of chloroquine upon the ultrastructure of pigmentary organelles in crayfish photoreceptor cells.

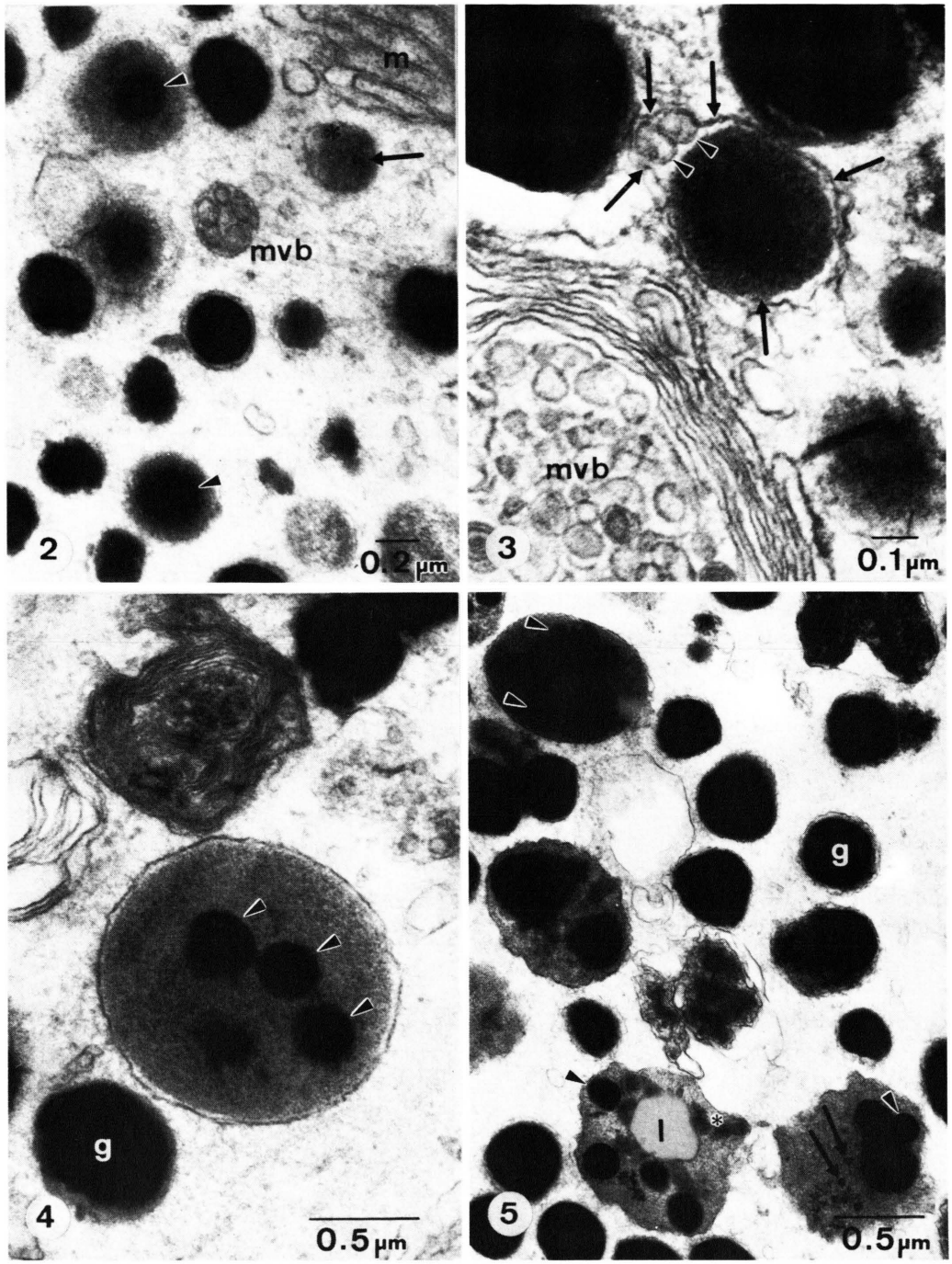


Table I. Mean values (± SD) of rhabdom size after irradiation with bright light and irradiation with bright light and additional chloroquine treatment.

Experimental condition	N° of retinae	N° of rhabdoms	Rhabdom length [μm]	Rhabdom width [μm]
Natural day-night light rhythm	4	17	107.6 ± 4.8	28.7 ± 2.3
Irradiated 9.2 mW/cm²/5 days	6	20	87.2 ± 8.2	15.7 ± 3.8
Irradiated 9.2 mW/cm²/5 days 0.5 mg chloroquine/day	6	25	97.2 ± 5.8	23.3 ± 2.9

in morphology and size to vesicles derived from photosensory membrane degradation (Fig. 3).

Just proximal of the basement membrane (topographic position 3, Fig. 1) lysosome-related bodies appeared in retinular cell axons (Fig. 4, 5) that contained a homogeneous matrix (Fig. 4) and small screening pigment-like granules that were smaller than the surrounding cytoplasmic screening pigment granules (Fig. 4, 5). Large MVB accumulated in retinular cell axons and almost completely filled the cytoplasm just proximal or distal of the basement membrane (topographic position 2 and 3, Fig. 1) (Fig. 6). Within the MVB, vesicles from photosensory membrane are degraded to a different degree. In some MVB homogeneous areas are visible (Fig. 6). Frequently such areas additionally reveal dense cores (Fig. 6). Large MVB often contained membrane delimited smaller MVB (Fig. 6, 7). Primary vesicles of these small MVB often were more degraded than the vesicles in the surrounding larger MVB (Fig. 7). In the proximal part of retinular cells (topographic position 4 in Fig. 1) large membrane-bound residual bodies (up to 3.5 μm in length) were observed in which screening pigment granules or remnants of them could still be detected (not shown).

In the axonal part of the retinular cells (between position 3 and 4 in Fig. 1) MVB were found in different stages of degradation of their content. These MVB contained frequently screening pigment-like granules (Fig. 8). In some of these MVB only photosensory membrane vesicles and screening pigment granules were observed (Fig. 8) whereas in others additionally an homogeneous matrix was present (Fig. 8, 9). Small, degraded MVB often showed characteristics of MVB (primary vesicles) and screening pigment granules (electron dense cores) (Fig. 9).

In the lamina ganglionaris (topographic position 5 in Fig. 1) glial cells identified by their glial

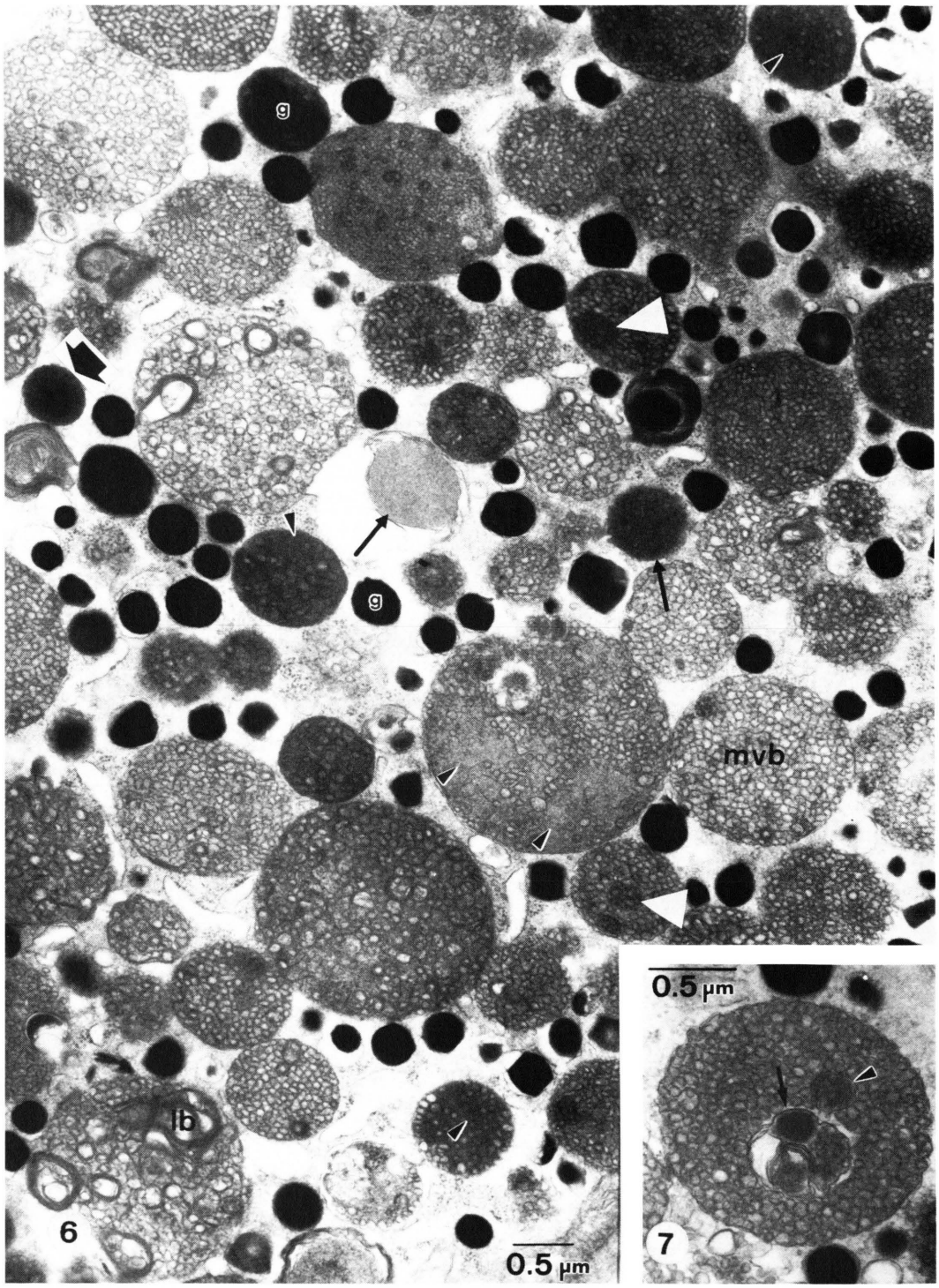
Fig. 2. After chloroquine treatment in combination with bright light exposure close to the rhabdomeral microvilli (m) small granules of different electron density are shown. Multivesicular bodies (mvb) of the same size as small screening pigment granules are present. Some of them that may have originated from degradation of MVB and reveal more or less homogeneous electron dense content (asterisk). In some organelles structures resembling degraded photosensory membrane vesicles can hardly be recognized (arrow). Frequently, granules with homogeneous content contain electron dense cores (arrowheads).

Fig. 3. A clear body with electron dense core is shown that is membrane surrounded (arrows). Between this clear body and its limiting membrane two vesicles can be detected (arrowheads) that are identical in size and morphology to the vesicles derived from photosensory membrane within a multi-vesicular body (mvb) shown in the lower left corner.

Fig. 4. In the axonal part of a retinular cell just proximal of the basement membrane (position 3, Fig. 1) an organelle of the size of MVB is shown that contains small screening pigment-like granules (arrowheads). These granules are smaller than the surrounding cytoplasmic screening pigment granules (g).

Fig. 5. In the same area as in Fig. 4 lysosome-related bodies are found in retinular cell axons which contain small screening pigment-like granules (arrowheads), membranous debris (asterisk), lipid droplets (l) and electron dense spots (arrows). These granules are smaller than surrounding cytoplasmic screening pigment granules (g).

Plate 2. Inhibiting effect of chloroquine of photosensory membrane degradation in crayfish photoreceptors.



fibers (Fig. 10) frequently contained screening pigment granules (Fig. 10). Macrophage-like cells of the optic neuropil revealed large abnormal vacuoles (not shown).

Discussion

Light-enhanced transductive membrane degradation in photoreceptors of invertebrates, as observed in this study, is in agreement with numerous earlier results [15, 17].

That the drug had reached the retinal tissue in the chloroquine treated crayfish was indicated by vacuolization and swelling of intracellular acidic compartments found in retinal macrophages. These changes are typical for weak bases such as chloroquine [30].

The effect of chloroquine on the ultrastructure of the crayfish retina was threefold:

First, pigmentary organelles appeared in MVB.

Second, MVB accumulated in retinular cell axons around the basement membrane. Accumulation of organelles below the basement membrane after slight illumination was also observed by Hafner *et al.* [8] in a white-eyed crayfish mutant lacking screening pigment. The third effect of chloroquine was inhibition of the light-induced degradation of photosensory membrane which was concluded from the slight reduction of rhabdom diameter.

The appearance of new morphologic features of pigmented organelles were not observed without chloroquine treatment [7, 31] or illumination [23]. The morphologic alterations seem not to be the consequence of new or abnormal pathways, induced by the drug, but of an inhibition of normal pathways. Normal pathways may have been slowed down, and intermediate forms of the lysosomal photosensory membrane degradation may have become visible. Such intermediate stages are the incompletely degraded MVB containing membrane vesicles, a homogeneous matrix and additional screening pigment granules (Fig. 9). In the absence of chloroquine, degradation of photosensory membrane is fast because MVB contained radioactivity already five minutes after injection of radio labelled leucin [32]. The rapidity of the membrane degradation may be the reason why the intermediate forms of this pathway are not observed when the lysosomal pathway is not inhibited by chloroquine.

The possibility that the dense granules were taken up from MVB by endocytosis is not likely for following reasons: They were not observed in MVB close to the rhabdoms, but only proximal of the rhabdoms in MVB in which degradation of their content had already begun. If the screening pigment-like granules had got into the MVB by endocytosis, this should have happened at sites where endocytosis of photosensory membrane occurs. This, however, was not observed.

Screening pigment granules within MVB regularly were smaller than the surrounding cytoplasmic screening pigment granules (Fig. 8). This finding is better explained by *de novo* synthesis than by endocytotic uptake. Pigmentary organelles within MVB were not observed without chloroquine treatment. Chloroquine blocks, and does not favour, endocytotic processes [28].

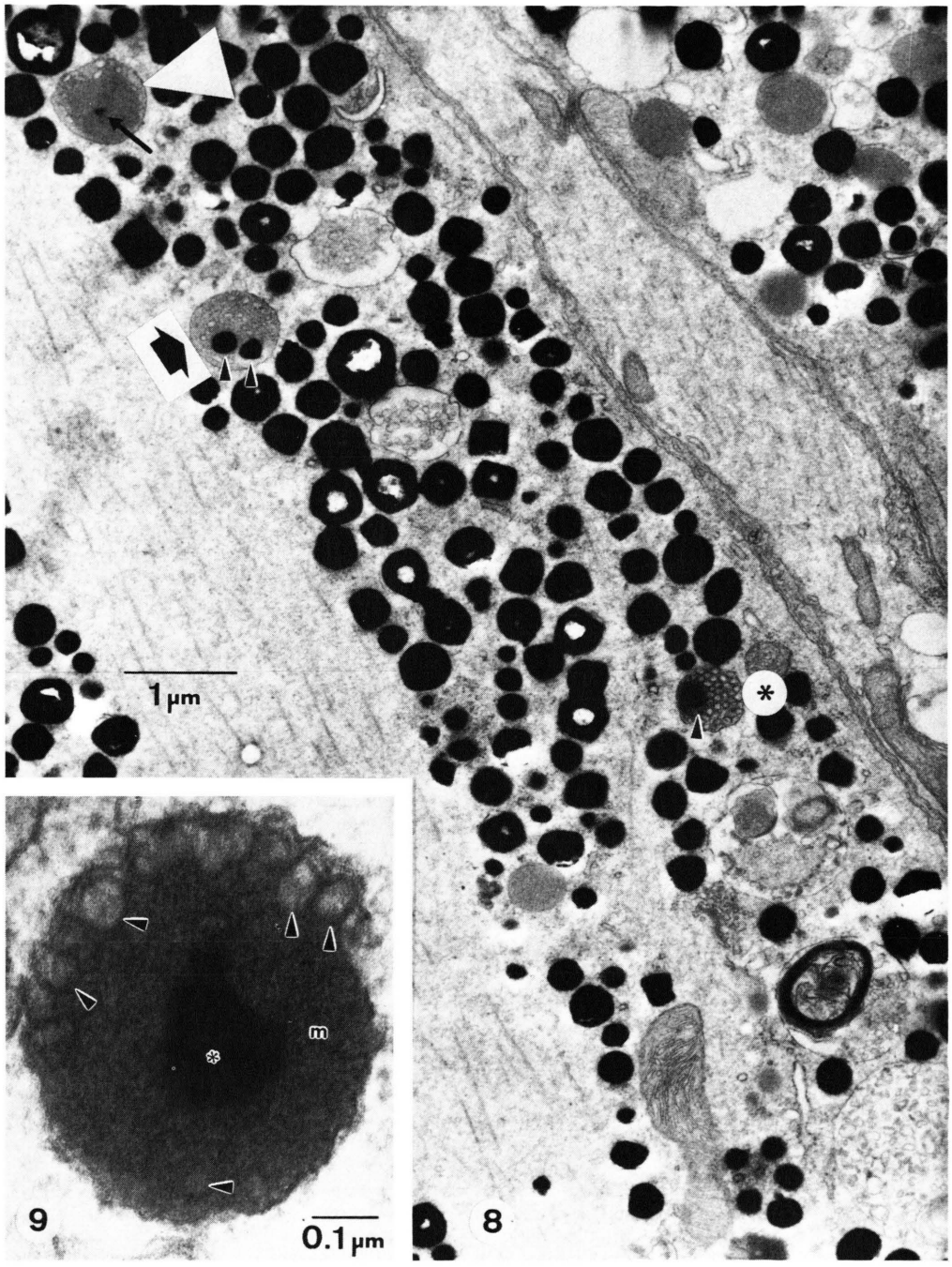
A second possibility is that the screening pigment-like granules are synthesized within MVB but are not of the chemical composition of screening pigment granules. However, this possibility is unlikely because normal residual bodies, for example those frequently found in macrophages of the crayfish optic neuropil, do not show the extremely high electron density of screening pigment granules. The same is true for residual bodies of macrophages of the rat [33].

So the possibility remains that the screening pigment-like granules might be synthesized within

Fig. 6. In longitudinally sectioned retinular cell axons proximal of the basement membrane (position 3, Fig. 1) multivesicular-bodies (mvb) or lamellar bodies (lb) accumulate after chloroquine treatment. Within the MVB, vesicles from photosensory membrane are degraded to a different degree. In some MVB homogeneous areas are visible (black arrowheads) whereas the content of others is completely homogeneous (small black arrows) or reveals dense cores (large black arrow). Large MVB can contain smaller ones (white arrowheads, see also Fig. 7). Screening pigment granules (g) are not found within MVB at this topographic position.

Fig. 7. A MVB contains a small, membrane delimited MVB (arrowhead). Inside this MVB the membrane vesicles are more degraded and are more electron opaque than the content of the large MVB. A second MVB inside the larger one contains lamellar structures, an electron opaque material (arrow) and vesicles.

Plate 3. Effect of chloroquine upon the ultrastructure of MVB in crayfish photoreceptors.



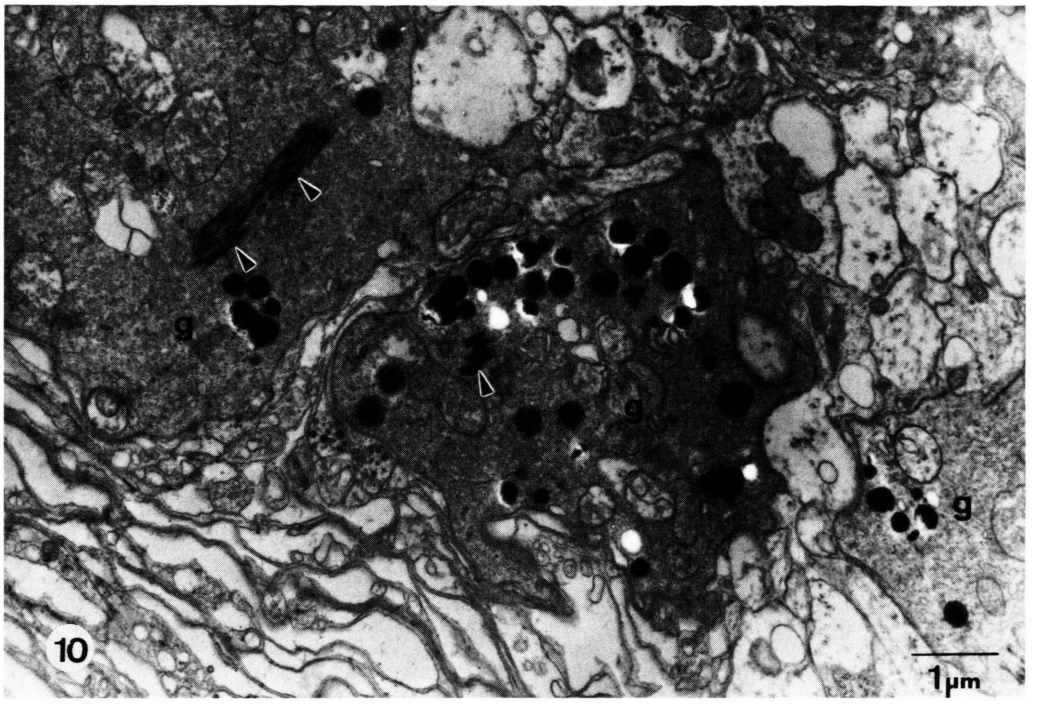


Fig. 8. Proximal of the basement membrane (between position 3 and 4 in Fig. 1) MVB at different stages of degradation of their content were found. These MVB frequently contained screening pigment-like granules (small black arrowheads). In some of these MVB, only photosensory membrane vesicles and screening pigment granules were observed (asterisk) while in others a homogeneous matrix was present in addition (large arrow). In MVB that contain only few vesicles and much homogeneous matrix (white large arrowhead) small electron dense spots in their centre (small arrow) are found which suggests the beginning of the formation of this electron dense material. In general screening pigment granules inside MVB were smaller than cytoplasmic screening pigment granules.

Fig. 9. MVB in the same topographic position as in Fig. 8 often show characteristics of MVB (primary vesicles, arrowheads) and screening pigment granules (electron dense cores, asterisk). These granules frequently reveal a homogeneous matrix (m).

Fig. 10. In the lamina ganglionaris (topographic position 5 in Fig. 1) glial cells (g) identified by their glial fibers (arrowheads) frequently contained screening pigment granules after chloroquine treatment.

MVB, such as shown in Fig. 9, and are not only morphologically but also chemically identical with cytoplasmic screening pigment granules. This idea is not unlikely because recently tyrosinase that is able to form xanthommatin *in vitro* was localized in lamellar bodies of photoreceptor cells [34]. Moreover it is known that xanthommatin is produced by degradation and occurs in excreta. Tryptophan is degraded quantitatively to kynurenine, 3-hydroxykynurenine and xanthommatin [35] during the metamorphosis of insects. Photoreceptor cells of invertebrates, like insects during metamorphosis, have to degrade high degrees of proteins from broken down rhabdomeres [11] and also form ommochromes.

Screening pigment granules were frequently observed in glial cells but were only rarely observed without chloroquine treatment. One reason for this finding may be inhibition of lysosomal degradation of these screening pigment granules by chloroquine. Presumably, these screening pigment granules had been exocytosed from photoreceptor cells. This indicates that screening pigment granules underlie turnover processes. However, how screening pigment granules are formed and which organelles are involved is still unknown.

The inhibiting effect of chloroquine upon light induced photosensory membrane degradation may be due to its influence on receptor mediated endocytosis. It is well known that chloroquine inhibits receptor-mediated endocytosis [28, 30] and degradative pathways involving lysosomal enzymes [36]. Photosensory membrane of arthropods is partly shedded into the extracellular space and then is taken up by the same cell by a process that resemble receptor-mediated endocytosis involving coated pits [15]. But such a receptor has not yet been localized or identified. If photosensory membrane endocytosis is indeed receptor-mediated, as seems to be the case, this would explain the inhibiting effect of chloroquine on light-induced rhabdom degradation. The mechanism of this inhibiting effect may be due to lack of the unknown receptor for endocytosis of aged photosensory membrane, because delivering of this receptor from the lysosomes and its recycling to the cell membrane might be inhibited.

The data of the present study support the idea that screening pigment granules in crayfish photoreceptors are formed in lysosomes that degrade photosensory membrane [27, 34].

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