Exocytic Clearing of Degraded Membrane Material From Pigment Epithelial Cells in Frog Retina

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Posterior eye cups of the frog Rana ridibunda were prefixed in the presence of tannic acid, osmicated and stained en bloc with uranyl acetate. Electron microscopy revealed the following features of membrane traffic in cells of the retinal pigment epithelium (RPE): (1) intracellular membranes with increased electron density undergo degradation within phagolysosomes and also outside lysosomes and are committed to be exocytized; (2) exocytized degraded membranes, organized as lamellar arrays, are evacuated through the basolateral extracellular space, through Bruch's membrane and into the choriocapillaris. Comparison of exocytic activity in animals kept either with a light cycle or in constant darkness suggests that exocytized lamellar arrays derive from membranes degraded through both lysosomal and non-lysosomal pathways. Autophagocytosis may contribute a substantial amount of material to exocytosis. On the other hand, exocytosis of heterophagosomes, if it takes place at all, involves only residual bodies at a final stage of degradation following slow proteolysis which is achieved within the RPE cells. Rapid transcytosis of heterophagosomes does not occur. Taken together, our observations suggest that, in the frog, exocytosis and subsequent evacuation of residual bodies is an efficient mechanism for clearing from worn-out organelles the metabolically highly active, non-dividing RPE cells. Invest Ophthalmol Vis Sci 28:2026-2037, 1987

Renewal of cellular constituents is a metabolically important phenomenon in cells which, under normal circumstances, do not undergo mitotic divisions. It implies both synthesis of molecules and organelles and degradation of worn-out constituents, in order to keep the cell volume constant. This perpetual replacement often follows a circadian rhythm and is particularly conspicuous in cells with high metabolic activity such as neurons, liver and muscle cells, as well as photoreceptor cells and cells of the retinal pigment epithelium (RPE). Both visual and RPE cells regularly replace their major cytoplasmic structures through a well-balanced system of new synthesis and autophagic digestion, in which autophagocytosis is a normal feature of the general metabolism. In addition, the two cell types form a polarized metabolic entity, with the photoreceptors rhythmically shedding the tips of their outer segments and replacing them through new synthesis. By phagocytizing and digesting the shed tips, RPE cells participate directly in the degradation of photoreceptor membranes. The periodicity of shedding and subsequent phagocytosis is given by a circadian rhythm which, in some species, can be synchronized by light. In the adult frog, the rhythm is directly related to the light cycle. Following ingestion by RPE cells, the newly formed heterophagosomes are digested by the lysosomal system. Concurrently, a massive mobilization of catabolic enzymes in these cells is observed. The disappearance of heterophagosomes from RPE cells towards the end of the light period and at the end of the dark period has led to the idea that they might be eliminated not only by enzymatic degradation, but also by rapid transcytosis and release into the basal extracellular space. That RPE cells may be capable of transcytizing ingested particles under experimental conditions has been demonstrated by presenting latex beads to the apical poles of the cells, both in situ and in vitro. Exocytized particles were found extracellularly at the basal side of the cells.

Exocytosis of membranous structures has been observed in various tissues. In type II alveolar epithelial cells of the lung and in Schwalbe line cells of the trabecular meshwork in the eye, exocytosis of phospholipid-rich arrays may have the common physiologic function of decreasing the surface tension. In the eye, this may facilitate streaming of aqueous humor through intercellular spaces in the anterior segment. In the liver and in the kidney, how-
ever, exocytosis of residual bodies was interpreted as being a consequence of high autophagic activity. Recently, yet another example of exocytosis of myelin-like figures, occurring in adipose tissue, has been briefly described. In this case, exocytosis appears to be due to lipolytic activity in the adipocytes.

In the present study, we describe the extracellular evacuation of multilamellar arrays from the basal side of the RPE through Bruch’s membrane towards the vascular system in the frog *Rana ridibunda*. Although we are not able to identify unambiguously the origin of the content of secondary lysosomes, there is evidence to suggest that most of the exocytized material derives from the degradation of cytoplasmic constituents of RPE cells. Transcytosis of phagocytized tips of photoreceptor outer segments may be limited to the expulsion of their residual bodies. Rhythmic exocytosis and evacuation towards the choriocapillaris may serve as an efficient way of clearing the RPE cells of incompletely degraded cellular constituents. Part of these investigations was reported in an abstract.

### Materials and Methods

#### Animals

Frogs of the species *Rana ridibunda* captured in Turkey and the Balkans were obtained through a local supplier and maintained, according to Swiss regulations that correspond to the ARVO Resolution on the Use of Animals in Research, in laboratory aquaria at 19°C in a 12 hr light/12 hr dark cycle during and for at least 5 days before experimental use. In some experiments, the dark period was extended to 19 hr in order to prevent shedding of rod outer segments.

#### Preparation of the Antiserum Against Photoreceptor Outer Segments

Dark-adapted frogs were decapitated, enucleated, and the retinas isolated from posterior eye cups under dim red light. For the isolation of photoreceptor outer segments, the procedure described by Adams et al was modified to deal with the small quantities of material recovered from frogs: Metrizamide gradients were centrifuged at 18,000 rpm for 20 min in a Beckman SW 41 rotor (Palo Alto, CA). As physiological solution, we used the Ringer solution described by Roof and Heuser: 10 mM Heps pH 7.4, 110 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 2 mM MgCl₂. Purified photoreceptor outer segments were kept as frozen pellets at −70°C until used for immunization or polyacrylamide gel electrophoresis.

Rabbits were immunized by intradermal injections at multiple sites in the back with approximately 1 mg of purified outer segments that were emulsified in Freund’s complete adjuvant. Boosters of 0.5 mg in Freund’s incomplete adjuvant were given at days 35 and 49. Blood was taken 1 week after the third injection. Total serum or an immunoglobulin fraction prepared on protein A-Sepharose were used.

The specificity of the antiserum was tested either by immunoblotting or by immunocytochemical localization (Fig. 1). Immunoblots of SDS-PAGE containing purified photoreceptor outer segments were done on 5–20% gradient gels essentially as described by Towbin et al. Antiserum was used at a dilution 1:300. As the second step, goat anti-rabbit immunoglobulins coupled to peroxidase and revealed by naphtholchloride were used to localize previously bound photoreceptor outer segment antibodies. Major proteins were identified by comparison with known molecular weight standards. cGMP phosphodiesterase and GTP-binding protein were identified by their selective extraction in hypotonic buffers and under specific light conditions.

#### Immunofluorescence Microscopy

Light-cycled frogs were sacrificed 2, 4, 6, 8 or 12 hr after onset of a light period. Enucleated posterior eye cups were processed by either of the two following methods: (1) Freeze substitution: eye cups were frozen by immersion in melting isopentane and transferred to absolute ethanol at −70°C. During freeze substitution for 1 week, the ethanol was changed once. After transfer to −35°C and then to 0°C, the tissue pieces were infiltrated with JB-4 (Polysciences, St. Goar, FRG) and embedded by slow polymerization on ice (cf. Fig. 6a, b). (2) Eye cups were fixed in 0.1 M phosphate buffer (pH 7.4) containing 2% paraformaldehyde and 0.1% glutaraldehyde for 3 hr at room temperature (cf. Fig. 6c). After washing in 75 mM cacodylate buffer (pH 7.4), the eye cups were treated with 50 mM NH₄Cl in cacodylate buffer (two times 15 min, 4°C), rinsed in cacodylate buffer, dehydrated at 0°C, and embedded in JB-4 as indicated above.

Semithin sections were incubated overnight at 4°C with the antiserum against photoreceptor outer segments (dilution 1:50). After rinsing in phosphate buffered saline (PBS), sections were treated with fluorescent goat anti-rabbit immunoglobulins (dilution 1:60; Behring Werke, Mannheim, FRG) for 1 hr at room temperature, rinsed extensively with PBS and embedded in Mowiol. Control sections were incubated with preimmune serum of the same rabbit. Preparations were viewed and photographed with a Zeiss photomicroscope equipped with epifluorescent illumination.
Transmission Electron Microscopy

Posterior eye cups were fixed at various times after onset of light (0, 2, 4, 6, 8, 12 hr) in 2.5% glutaraldehyde in 75 mM phosphate buffer, pH 7.0, containing 3 mM MgCl₂ and 2 mg/ml tannic acid (Mallinckrodt Inc., Paris, KY). After 15 min, the eye cups were cut into small pieces and fixed further in the same fixative for 3 hr at room temperature. The pieces were then washed in 75 mM cacodylate buffer (pH 7.4, 4°C) and postfixed in a solution of 1% aqueous osmium tetroxide and 1.5% potassium ferrocyanide for 1 hr (4°C) and stained en bloc in 2% uranyl acetate (1 hr, 4°C). After rapid dehydration in ethanol, the tissue was embedded in Epon. Thin sections were stained in lead citrate and viewed with a Philips 300 electron microscope (Eindhoven, Holland).

Immunoelectron Microscopy

For the visualization of determinants derived from photoreceptor outer segments in phagosomes, we used the protein A-gold technique on Lowicryl K4M thin-sectioned material (Chemische Werke Lowi, Waldkraiburg, West Germany). Colloidal gold particles were prepared according to Frens and coupled to protein A. The tissue was fixed in 2% paraformaldehyde, 0.1% glutaraldehyde in 75 mM phosphate buffer (pH 7.4) for 2 hr at room temperature, washed twice in the same buffer and once in 75 mM cacodylate buffer (pH 7.4) before quenching in 50 mM NH₄Cl in cacodylate buffer. Dehydration in ethanol was performed at low temperature as indicated for embedding in Lowicryl K4M. Thin sections were preincubated with 0.5% ovalbumin in PBS for 5 min before incubation in antisera against photoreceptor outer segments (dilution 1:100 or 1:200) in the presence of ovalbumin. The incubation was done overnight at 4°C, then for 2 hr at room temperature. Protein A-gold complexes were applied for 1 hr at room temperature. Sections were stained in uranyl acetate and lead salts. Control sections were incubated in preimmune serum instead of specific serum, or serum incubation was omitted and sections incubated in protein A-gold only.

Results

Characterization of the Antiserum

On immunoblots, the rabbit antiserum against photoreceptor outer segments reacts with at least four different proteins, namely rhodopsin, cGMP phosphodiesterase and two undetermined minor proteins (Fig. 1a). Apparently, the serum does not contain any antibodies against GTP-binding protein and large integral membrane protein. We did not check for the presence of antibodies against lipid components in this serum.

Immunofluorescence microscopy showed antiserum staining only of rod outer segments and phagosomes containing outer segment membranes within the RPE (Fig. 1 b-d). The high specificity of the antiserum was confirmed by immunoelectron microscopy (Fig. 1c, g); 3 hr after onset of light, immunogold labeling was confined to the outer segment, whereas the inner segment did not react with the antiserum. The preimmune serum did not display any reaction, either by immunoblotting (Fig. 1a), by immunofluorescence (Fig. 1d) or by immunoelectron microscopy (Fig. 1f).

Faint but distinct fluorescent staining with the antiserum was observed on rod inner segments in material that had been fixed at the end of a 12 hr light period (cf. Fig. 6c). In addition, Figure 6c shows two weakly stained cones. Since such staining was not observed earlier in the light cycle, it may indicate new synthesis within the inner segment of some of the antigens revealed by our antiserum rather than being an artefact of aldehyde fixation. Staining of cones suggests that some of the antigens are common to both photoreceptor cell types.

Degradation of Cytoplasmic RPE Constituents

Throughout both the light and the dark period of a daily cycle, autophagic vacuoles were observed scattered throughout the whole body of the epithelial cells (Fig. 2a). They contained endoplasmic reticulum, Golgi stacks and other vesicular structures, mitochondria and, rarely, melanin granules. Some of these vacuoles were associated with lipid droplets. As exemplified in Figure 2b and c, their size, their content and the degree of impregnation with tannic acid varied considerably. The largest vacuole visible in Figure 2b does not show increased contrast. Its content is still recognizable as a portion of nondegraded cytoplasm containing endoplasmic reticulum and other vesicular structures, which suggests that it is newly formed. The content of the neighbouring vacuoles is degraded beyond recognition and appears as lamellar-like whorls and granular constituents. Their high electron density is due to impregnation with tannic acid and contrasts sharply with apposing membranes of the smooth endoplasmic reticulum (Fig. 2c).

Membranes with increased electron density were not always confined to the phagolysosomal vacuoles. They were seen also in smooth endoplasmic reticulum in patches contiguous with normally stained membranes (Fig. 2d, e); some mitochondria with
Fig. 1. Characterization of a rabbit antiserum against photoreceptor outer segments. (a) Immunoblots of purified outer segments of *Rana ridibunda* using either immune or preimmune serum. Proteins were separated on SDS-PAGE. RHO-1-4: mono- and oligomeric rhodopsin. GBP: GTP-binding protein. PDE: cGMP phosphodiesterase. LIP: large integral membrane protein. Dark dots: molecular species against which antibodies were made. Open circles: major proteins against which no antibodies had been produced. Arrowhead: minor protein that is not visible by Coomassie blue staining, but against which antibodies had been produced. First and third slot: Coomassie blue-stained gels after transfer. Second and fourth slot: Nitrocellulose blots after incubation with immune and preimmune serum, respectively. (b–d) Immunofluorescence microscopy of semithin sections of the outer retina of *Rana ridibunda*. (b) Phase contrast of (c). (c) Section incubated with immune serum shows specific staining of outer segments and heterophagosomes (PH). (d) Control section incubated with preimmune serum. Arrowheads point to erythrocytes in choriocapillaris which display faint autofluorescent staining. (e–g) Immunoelectron microscopy using the protein A-gold technique on Lowicryl K4M embedded thin sections. Staining of outer segments (OS) with immune (e, g) and preimmune (f) serum, respectively. (g) Specific staining is confined to the outer segment of the photoreceptor cells, whereas the inner segment (IS) is not labeled. RPE apical region of retinal epithelial cell. Bars, (b–d) 20 μm, (e–g) 1 μm.

Exocytosis of Degraded Cellular Constituents

Residual bodies, composed of concentric, densely stained layers forming large lamellar arrays, may be considered as the end product of hydrolytic degradation. Frequently, they were in close proximity to the lateral extracellular space. Sometimes, fusion of the plasma membrane with the membranes of these arrays was observed (Fig. 3a), which is indicative of imminent exocytosis.

The bulk of exocytized material was found within the basolateral portion of the extracellular space (Fig. 3b) and only occasionally, fragments were observed within its apical portion (cf. Fig. 2a). The lamellar
Fig. 2. Degradation of cytoplasmic constituents in RPE cells. (a) Numerous autophagic vacuoles (AV) are scattered throughout the cytoplasm. A heterophagosome (PH) is located within the basal cellular compartment. Note a patch of extracellular material stained with tannic acid (arrowhead). BM Bruch's membrane, MB myeloid bodies, ZA zonula adherens, N nucleus, LS lateral intercellular space, SRS subretinal space, LI lipid droplet. (b) Autophagic vacuoles with granular and lamellar content at different stages of degradation. Arrows point to the membrane of a freshly formed vacuole. Note high density of tannic acid staining of vacuoles at advanced stage of degradation as well as of the lateral intercellular space (LS). ME melanin granule. (c) Autophagic vacuole (arrows) with densely stained peripheral lamellae. Smooth endoplasmic reticulum (SER) and mitochondria (MI) do not show increased staining with tannic acid. (d, e) Densely stained patches of the SER are in connection (arrowheads) with faintly stained SER. (e) Membranes of myeloid body (MB) are in connection with strongly stained convoluted membranes. (f) One of the mitochondria (MI) shows signs of degradation (arrowhead) without being included in an autophagic vacuole. Bars, 1 μm.
Fig. 3. Exocytosis and evacuation of tannic acid-stained lamellar material. (a) Residual bodies are in connection (arrows) with the intercellular lateral space (LS). (b, c) Exocytized material fills the intercellular basolateral space. IF basal infolding, JC junctional complex. (c) Note endocytic activity of the basal plasmalemma (arrowheads). (d) Densely packed lamellar arrays are deposited (arrow) between the basal plasmalemma and the basal lamina (BL). LI lipid droplet, BM Bruch's membrane, EN endothelial cell. The precipitate visible in (a) is observed occasionally upon inclusion of tannic acid in the fixative. Bars, 1 μm.

bodies appeared as loosely associated arrays in the vitreal portion of the basolateral space and as condensed small fragments in the basal portion, where they completely filled the space between the infoldings (Fig. 3b, c). The fragments appeared to be lined up between the plasmalemma and the basal lamina (Fig. 3c, d).

The deposition of exocytized material on the basal side of the RPE occurs locally and leads to massive accumulations within the inner collagenous zone of Bruch's membrane (Fig. 4a). Preferential outlets towards the choriocapillaris are provided by local gaps in the elastic layer. Moreover, the intimate apposition of extended endothelial protrusions (Fig. 4b) and the
Fig. 4. Accumulation of exocytized material and its release into the capillaris. (a) Accumulation of small packets of membranous lamellae between the infoldings (arrows) at the basal side of the epithelial cell and between the plasmalemma and the basal lamina (BL) (arrowhead) bulging out towards Bruch's membrane (BM). Note the passage of material through a gap in the elastic layer (EL). LS lateral intercellular space. (b) Higher magnification of accumulated material within Bruch's membrane, where lamellipodia of the endothelial cell (EN) are in intimate contact with the tannic acid stained material. (c-e) Passage between the endothelial cells into the lumen of the capillary (CA). RPE retinal epithelial cell. Bars, 1 μm.

Exocytized material is suggestive of an active role of the endothelial cells in guiding the fragments through their intercellular spaces towards the lumen of the capillary (Fig. 4c–e). Figure 5 gives an overview of the basal region of the RPE and adjacent Bruch's membrane, where exocytosis occurs simultaneously in a group of neighbouring cells. The oblique section plane gives some insight into the topology of the exocytized material accumulating between the basal infoldings of the epithelial cells and in particular within the inner collagenous zone of Bruch's membrane. Fragments are seen passing through the endothelial layer of the capillary.

During late hours of the light period, when exocytosis is prominent, an estimated 10% of RPE cells in an eye cup preparation evacuate simultaneously exocytized residual material. Yet, exocytic activity is maintained also during the dark period as well as during prolonged darkness (up to 19 hr), when shedding of rod tips had been prevented.

**Origin of Exocytized Material**

Attempts to determine the origin of exocytized material are complicated by the fact that, in RPE cells, two lysosomal pathways run in parallel. On the one hand, autophagic breakdown of cellular constituents is a frequent and normal event (cf. Fig. 2). On the other hand, rhythmical degradation of shed and phagocytized photoreceptor disks leads to the formation of heterophagosomes. Auto- and heterophago-
Fig. 5. Oblique section through the basal side of RPE cells and adjacent Bruch’s membrane (BM). The view is a mount of several micrographs and shows the simultaneous accumulation and evacuation of exocytized tannic acid-stained lamellar arrays towards the choriocapillaris (CA). Some material (arrowheads) is found within the lateral intercellular space (LS) and between the basal infoldings (IF). Alignment of similar material is observed along the basal lamina (BL) (small arrows), whereas the bulk is located within the inner collagenous zone of Bruch’s membrane. Caps (asterisks) in the elastic layer (EL) are discernible. Some material is released into the lumen of the capillary (open arrow). EN endothelium, LI lipid droplet. Bar, 10 μm.

Autophagosomes appear to be spatially separated: autophagosomes are scattered throughout the whole cell body, and heterophagosomes, following ingestion, are transported rapidly into the base of the cell. Therefore, exocytized material found within the intercellular lateral space during late hours of the light period may have come from autophagosomes rather than from heterophagosomes. To test this hypothesis, we have tried to prevent shedding and subsequent phagocytosis of photoreceptor outer segments (in particular of rods) by omitting the dark-to-light transition of the light cycle. In animals that had been kept in prolonged darkness (19 hr), we observed exocytized lamellar arrays within the intercellular lateral space (not shown) comparable to the situation found in normally cycled specimens (cf. Fig. 3). This suggests that such exocytized residual bodies are derived from autophagic breakdown of RPE constituents.

We also followed the proteolytic degradation of photoreceptor outer segments contained in heterophagosomes. Heterophagosomes were stained immunocytochemically using the antiserum prepared against photoreceptor outer segments (cf. Fig. 1). By immunofluorescence microscopy, we regularly found immunoreactive phagosomes 6 to 8 hr into the light cycle, and some were still immunoreactive even after 12 hr (Fig. 6). This suggests that not all antigenic determinants of rod material are degraded by the time of the onset of the dark cycle. Immunoreactive phagosomes were found exclusively within the epithelial cells (Fig. 7a–c). As the phagosomes fragmented and decreased in size, immunolabeling became patchy (Fig. 7b). Assuming comparable accessibility of the epitopes within the whole organelle, this means that the degradation of the phagosome is compartmentalized. No immunolabeling was ever found outside of the RPE cells, either between the basal infoldings or at the level of Bruch’s membrane (Fig. 7d, e). Although reminiscent of a heterophagosome containing shed photoreceptor disks, the extracellular lamellar body depicted in Figure 7d does not show any reaction with the antiserum and thus cannot be traced back to its origin. Moreover, exocytized fragments lying within the lateral extracellular space and along the basal lamina were not immunoreactive (Fig. 7e). Taken together, these observations show that the proteolytic degradation of heterophagosomes containing photoreceptor disk material proceeds slowly and within the RPE cells and is not terminated at the end of a light period. Rapid exocytosis of heterophagosomes at an early stage of degradation is therefore unlikely.
Fig. 6. Immunofluorescence microscopy on semithin sections through the outer retina. Photoreceptor outer segments and heterophagosomes (arrows) are specifically stained with antisera against photoreceptor outer segments. Retinas were fixed 6 hr (a), 8 hr (b) and 12 hr (c) after onset of light. (e) Some of the phagosomes containing shed rod outer segments are still immunoreactive at the end of the light period (12 hr). Note the difference in staining intensity in (a) and (b), fixed by freeze substitution, and (c), fixed by an aldehyde mixture. (c) Faint, but specific staining over background of rod inner segments and cones (CO). Bar, 10 μm.

Discussion

The present study illustrates the importance of the elimination of membranous material by exocytosis from frog RPE cells. Membranous arrays, especially when they are located outside the cells, have a high electron density upon stabilization of phospholipids by treatment with tannic acid-osmium tetroxide and en bloc treatment with uranyl acetate. This increased contrast has allowed us to observe heterogeneities in intracellular membranes, the expulsion of membrane arrays from RPE cells, their massive accumulation within the basolateral extracellular space and their final release into the vascular system.

Elimination of cellular components through exocytosis is a rather uncommon process in higher organisms. Up to now, it has been documented in proximal tubular cells of the kidney and in hepatocytes. In liver cells, exocytosis is thought to be a consequence of the discharge of lysosomal contents derived from extremely active autophagocytosis. In the RPE, the situation is more complex, since lysosomal contents may come from two different phagocytic pathways, namely from autophagocytosis and from heterophagocytosis of photoreceptor outer segments. Since, in lysosomes, proteolysis is in general more rapid than lipid degradation, the lipid content of secondary lysosomes at an advanced stage of degradation cannot be traced back unambiguously to its origin, although its increased staining by tannic acid indicates a general enrichment in saturated phospholipids and particularly in phosphatidyl choline. Early after sequestration, however, the two lysosomal populations are still distinguishable by morphological, spatial and immunocytochemical criteria. In our study, heterophagosomes were identified by their staining with antibodies directed against proteins of photoreceptor outer segments. Some of them were still immunoreactive at the end of a 12 hr light cycle, indicating that proteolysis is rather slow. Proteolytic degradation of specific determinants takes place entirely within the RPE, and no immunostaining could be observed on any material outside the epithelial cells. These observations argue against the possibility that heterophagosomes are eliminated by rapid transcytosis, a mechanism proposed to bypass lysosomal degradation. However, without selective markers for the content of advanced secondary lysosomes, we cannot exclude that part of the exocytized material consists of undigested remnants of heterophagosomes that, once proteolysis has been achieved, are evacuated into the extracellular space. In general, however, it seems that the contribution of heterophagosomes to the amount of exocytized material is minor and that photoreceptor disks are efficiently degraded by the lysosomal system, since the enzyme complement of RPE lysosomes appears to be highly specialized for the degradation of rhodopsin and phospholipids. On the other hand, our observations suggest that a significant part of the exocytized mate-
Fig. 7. Immunogold labeling of RPE with antiserum against photoreceptor outer segments. (a–c) Specific labeling of heterophagosomes (PH) within the epithelial cells. (a) Labeling intensity of an early (3 hr after shedding) heterophagosome is equal to that of rod outer segment (OS). N nucleus. (b) Patchy distribution of gold particles on a fragmenting heterophagosome (3 hr after shedding) may reflect its progressive enzymatic degradation. ME melanin granules, MI mitochondria, BM Bruch's membrane, BL basal lamina. (c) Late immunoreactive heterophagosome (12 hr after shedding) in close apposition to a lipid droplet (LI). (d) Unlabeled membranous whorl in connection with the extracellular space (arrows) may represent either the residual body of a degraded heterophagosome or the remnants of a late autophagic vacuole. (e) Fragments of exocytized material (arrowheads) within the basolateral space (arrows) and along Bruch's membrane (BM) are unlabeled. Bars, 1 μm.
rial derives from autophagic breakdown: in frogs that had been kept in prolonged darkness, shedding and subsequent phagocytosis of rod outer segments is shut off. Although we cannot completely exclude the possibility that residual bodies of heterophagosomes that have been ingested the day before have been expelled into the extracellular space, the bulk of exocytized material that we observed in dark-adapted animals must come from autophagosomes. In addition, some of the exocytized material may come from non-lysosomal degradation of cytoplasmic membranes of RPE origin. Indeed, as discussed below, the use of tannic acid has provided evidence that this process takes place in RPE cells.

Using tannic acid of relatively low molecular weight, we have observed an increase in electron density not only of extracellular constituents, but also of distinct intracellular RPE organelles, in particular of advanced phagolysosomes and of membrane segments of smooth endoplasmic reticulum and mitochondria that were not in phagocytic vacuoles (cf. Fig. 2). A plausible explanation for this staining heterogeneity of RPE inner membrane systems outside lysosomes involves local activity of different phospholipases. Interestingly, the membrane-associated forms of phospholipases A1 and A2, with alkaline pH optimum and calcium-dependent activity, digest phosphatidyl ethanolamine more efficiently than phosphatidyl choline, latter of which is digested preferentially at low pH and independently of calcium, probably in lysosomes. The substrate preference of activated membrane-bound phospholipases for phosphatidyl ethanolamine could thus lead to local enrichment of phosphatidyl choline in certain membrane segments, which are recognizable by their increased electron density upon tannic acid staining. Hence, in the special case of RPE cells, we may consider the high contrast induced by tannic acid as strong morphologic evidence for ongoing non-lysosomal degradation and, eventually, for commitment of such membrane segments to exocytosis. It is not altogether clear, however, how much of the exocytized material is contributed by this non-lysosomal degradative pathway.

In conclusion, we have documented in the frog the existence of an exocytic pathway of membranous arrays from the RPE into the vascular system. Some of our observations suggest that the exocytized material is enriched in phosphatidyl choline and represents either residual bodies from autophagic and heterophagic lysosomal pathways, or remnants of organelle membranes digested by non-lysosomal, membrane-bound hydrolases. These two processes appear to entail appreciable exocytosis also in dark-adapted animals, when shedding of rod outer segments and ensuing heterophagocytosis are arrested. It seems reasonable to assume that exocytosis represents a potent mechanism for clearing the non-dividing RPE cells of indigestable membrane residues, regardless of their origin. This would explain why, in the frog, lipofuscin deposits have never been detected. Investigating exocytic processes in the rat RPE, we have observed that their incidence is rather low compared to the frog RPE (Y. Gambazzi and E. Rungger-Brändle, unpublished observations). It would be interesting to know whether low exocytic activity in mammals may lead to lipofuscin deposition during aging.

Key words: autophagocytosis, heterophagocytosis, immunolocalization, tannic acid, ultrastructure

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