Acidification of Phagosomes and Degradation of Rod Outer Segments in Rat Retinal Pigment Epithelium

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Purpose. The authors investigated the phagocytic processes of the rod outer segments (ROS) in rat retinal pigment epithelium (RPE) cells, and the appearance of lysosomal enzymes, acidification, and degradation of the contents in the phagolysosomes. In particular, they examined the effect of bafilomycin A₁, a specific inhibitor of vacuolar-type H⁺-ATPase, on the degradation of ROS in the RPE cells in vivo.

Methods. A lysosomal enzyme (cathepsin D), a lysosomal membrane protein (LGP107), and opsin were localized in the RPE cells by the immunogold electron microscopic technique. Bafilomycin A₁ was injected into the vitreous, and acidification of the phagosomes was measured in vivo by injecting 3-[2,4-dinitroanilino]3′amino-N-methyldipropylamine (DAMP) in the vitreous and detecting the accumulation of DAMP in the phagolysosomes using anti-dinitrophenol antibody.

Results. Opsin was abundantly detected in phagosomes that did not contain cathepsin D, but the immunolabeling of opsin rapidly disappeared soon after the appearance of cathepsin D. By double staining with cathepsin D and DAMP, it was shown that the pH of the phagosomes dramatically decreased after fusion with lysosomes. When bafilomycin A₁ was injected into the vitreous, many large phagolysosomes containing cathepsin D appeared in the RPE cells, in which the immunoreactivity of opsin was well preserved.

Conclusions. Degradation of opsin and acidification proceeded almost parallel with the appearance of cathepsin D in the phagolysosomes. Bafilomycin A₁ did not inhibit the fusion of phagosomes with lysosomes, but it increased intraphagosomal pH and markedly inhibited the degradation of ROS in the phagolysosomes. This result indicates that vacuolar-type H⁺-ATPase is essential for acidifying the lumen of phagolysosomes and subsequent protein degradation of ROS in the RPE cells. Invest Ophthal Vis Sci. 1994; 35:568-579.

The retinal pigment epithelium (RPE) is a single layer of cells that lies beneath the neural retina and plays important roles in the maintenance of the neurosensory retina,¹⁻³ phagocytosis of the rod outer segments (ROS) being one of the most important ones. Phagocytosis of the shed ROS occurs particularly in relation to the light cycle.⁴⁻⁷ La Vail⁴⁻⁶ found that in albino rats reared on a 12-hours light–12-hours dark schedule, the ROS disk shedding followed a circadian rhythm, with a burst of shedding and phagocytosis occurring between 30 minutes and 2.25 hours after the onset of light.

Phagocytosis of the ROS consists of multiple processes—recognition and binding, ingestion and formation of phagosomes, and degradation.⁸⁻¹³ Once the ROS are ingested and phagosomes formed, lysosomes fuse with them to deliver lysosomal enzyme into the phagosomes.¹⁴ Then digestion proceeds in the phagolysosomes. Intraphagosomal pH is also thought to decrease during this fusion process. The relationship of the acidification and the appearance of lysosomal enzymes, however, is not yet clearly known.
Acidification of Phagosomes in RPE

Using pH-sensitive dyes, several investigators have demonstrated progressive acidification of the phagosomal and lysosomal compartments to levels as low as pH 5.5 to 6.5 and pH 5.1 to 5.5, respectively. For electron microscopic determination of intracellular acidic compartments, Orci and Anderson introduced the use of 3-[2,4-dinitroanilino]3’-amino-N-methylpropylamine (DAMP), a weak base that contains a dinitrophenol group for easy detection with an antibody. DAMP accumulates in acidic compartments, depending on the H+ concentration, and is retained quantitatively after fixation so the pH of acidic compartments can be measured using the immunogold labeling technique.

Vacuolar-type H+-ATPase (V-ATPase) is localized in organelles of the central vacuolar system such as coated vesicles, chromaffin granules, the Golgi complex, lysosomes, neurosecretory granules, and fungal vacuoles. This suggests that V-ATPase is responsible for maintaining the acidic environment in these compartments. It has been suggested that the acidification in phagosomes or phagolysosomes is due to the V-ATPase that may be supplied from acidosomes or lysosomes to phagosomes. In fact, acidification of phagosomes in macrophages has been shown to depend on V-ATPase.

Bafilomycin A1, a macrolide antibiotic isolated from Streptomyces griseus, is a potent and highly specific inhibitor of the V-ATPase at nanomolar concentrations. However, it does not inhibit other types of ATPase, such as F1,F0-ATPase and E1,E0-ATPase in vitro. This drug is also effective on cultured living cells.

In this study, we immunoelectron microscopically analyzed the phagocytic process in detail, detecting a lysosomal enzyme (cathepsin D), a lysosomal membrane protein (LGP107), and opsin, and we determined the intracellular pH of the RPE cell by injecting DAMP in the vitreous. We have shown that degradation of opsin and acidification proceeded in good correlation with the appearance of cathepsin D in the phagolysosomes. To clarify the biologic significance of the acidification of phagolysosomes in the RPE cells, we injected bafilomycin A1 into the rat vitreous soon after onset of light. Bafilomycin A1 did inhibit the acidification of phagolysosomes, and the degradation of the ROS was significantly inhibited by this antibiotic.

METHODS

Animals

Male albino Sprague-Dawley rats (8 to 10 weeks) were obtained from a local supplier. They were given a commercial chow and water ad libitum and were maintained under controlled light–dark conditions (12 hours light–12 hours dark). The present investigations conformed to the ARVO Resolution on the Use of Animals in Research.

Antibodies Against Cathepsin D and LGP107

Antibodies against rat spleen cathepsin D and rat LGP107 were prepared as reported by Yamamoto et al. and Furuno et al.

Preparation of Anti-Opsin Antibody

The ROS membranes were prepared from bovine retinas by sucrose density gradient centrifugation, as reported by Makino et al. The membranes were solubilized with detergent (4% L-1690), and rhodopsin was purified with concanavalin A-Sepharose and DEAE-cellulose columns and was subjected to SDS-PAGE (10% gel). The opsin band with an apparent molecular mass of 35 kDa was cut from the gel. The gel fragments were ground with a mortar and suspended in phosphate-buffered saline (PBS), pH 7.4. Japanese white rabbits were immunized with 1 ml emulsion of the gel suspension (~200 μg opsin) mixed with Freund’s complete adjuvant. Three weeks later, the rabbits were boosted with the same amount of opsin mixed with Freund’s incomplete adjuvant, and 2 weeks later antisera were obtained. The immunoglobulin fraction was purified by ammonium sulfate fractionation and DEAE-column chromatography. Bovine opsin was coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden), and antibodies against bovine opsin were further purified by the opsin-Sepharose column.

Injection of Bafilomycin A1, Chloroquine, or Nigericin Into the Vitreous

Rats were anesthetized with ether, and 10 μl of either 10 μM or 100 μM bafilomycin A1 (Wako Pure Chem Indst, Osaka, Japan) in 10% DMSO was injected into the vitreous body using a Hamilton microsyringe (50 μl) with a 27-gauge needle soon after the onset of light at 8 AM. As a control, 10 μl of 10% DMSO in PBS was injected into the opposite eye of the same rat (mock injection). Chloroquine (10 nM dissolved in PBS) or nigericin (50 μg/ml dissolved in PBS containing 1% ethanol) was injected into the vitreous body similar to bafilomycin A1.

Fixation of the Tissue

Rats were anesthetized with ether and perfused through the left ventricle with Hank’s solution, pH 7.4, for 3 minutes, then fixed by perfusion with 4% paraformaldehyde containing 1% glutaraldehyde in Hank’s solution, pH 7.4, for 10 minutes and washed with PBS containing 50 mM NH4Cl for 5 minutes. Specimens were obtained from the equatorial area of the retina. For conventional electron microscopic observation, the eyes were further fixed with 4% para-
formaldehyde containing 3% glutaraldehyde in Hank’s solution (pH 7.4) for 3 hours and subsequently fixed with 1% OsO₄ in 0.05 M cacodylate buffer, pH 7.4, for 1 hour. Approximately 15 minutes elapsed from the time of anesthesia until the time RPE was fixed by perfusion.

Conventional Electron Microscopic Observation

The fixed retina was embedded in epon after dehydration in a graded series of alcohol. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and observed with a Hitachi (Tokyo, Japan) H-7000 electron microscope. The number of phagosomes per μm² of the cytoplasm of the RPE cells and the area of phagosomes in the RPE cells (% of cytoplasm) were measured. The average area of cytoplasm that we measured in each experiment was about 400 μm².

The areas of phagosomes, lysosomes, and cytoplasm of the RPE cells were measured on electron micrographs by a graphic digitizer (SD-311, Wacom, Tokyo, Japan) connected to a personal computer (PC-9801 CV, NEC, Tokyo, Japan).

Electron Microscopic Localization of Cathepsin D and LGP107 by the Protein A-Gold Technique on Frozen Ultrathin Sections

Frozen ultrathin sectioning was carried out as described by Tokuyasu with some modification. The fixed retina was cut into small pieces, incubated overnight in 2.3 M sucrose in 0.1 M sodium phosphate buffer (pH 7.4) containing 20% polyvinyl pyrrolidone, and rapidly frozen in liquid propane at −180°C. Frozen ultrathin sections were cut with a Reichert (Wien, Austria) Ultracut N with a cryoattachment (FC-40) at −115°C to an approximate thickness of 70 to 80 nm. The sections were picked up on formvar-carbon-coated nickel grids, incubated with 2% gelatin in PBS containing 0.3% Triton X-100 for 30 minutes, and incubated for 30 minutes with 0.5% BSA. After washing six times with gelatin solution, and finally treated with protein A-gold complex (4 nm in diameter, OD 525 nm = 0.023) for 30 minutes. After washing with sodium cacodylate buffer (pH 7.4), sections were post-fixed in 2% glutaraldehyde and then in 1% OsO₄, stained with uranyl acetate, embedded in LR White resin, and observed with a Hitachi H-7000 electron microscope.

Immunogold Localization of Opsin and Cathepsin D on LR White Sections

The fixed retina was dehydrated in a series of graded ethanol and embedded in LR White at −20°C. The resin was polymerized in a UV-ray polymerizer TUV-200 (Dosaka EM, Kyoto, Japan). Ultrathin sections were cut, incubated with 0.5% bovine serum albumin (BSA) in PBS for 5 minutes, and incubated for 30 minutes with anti-opsin antibody (140 μg/ml) or with anticytetestin D antibody (50 μg/ml) in PBS containing 0.5% BSA. After washing in PBS containing 0.5% BSA, the sections were incubated for 15 minutes with protein A-gold complex (8 nm diameter, OD 525 nm = 0.08). The sections were then washed with 0.1 M cacodylate buffer (pH 7.4), postfixed with 2% glutaraldehyde for 10 minutes, and stained with uranyl acetate and lead citrate.

Detection of Acidic Compartments by DAMP Procedure

DAMP, 1 mg/ml in 80% ethanol solution (Oxford Bio- medical, Oxford, MI) and DAMP * 2HCl (Dojin Lab, Kumamoto, Japan) were used in this study, and similar results were obtained by both probes. Rats were anesthetized with ether, and 10 μl of 1.6 mM DAMP or DAMP * 2HCl dissolved in PBS was administered into the vitreous at 6 PM. The next day, rats were fixed by perfusion as described above.

For light microscopic study, we performed the avidin-biotin-peroxidase complex (ABC) method (Vectastain, Vector Labs, Burlingame, CA) on cryosections. Eyes were enucleated and frozen in liquid nitrogen. Frozen sections were cut with a cryostat and mounted on gelatin-coated glass slides. The sections were preincubated for 30 minutes with 0.3% H₂O₂ in PBS to inactivate the endogenous peroxidase activity and for 15 minutes with PBS containing 20% normal goat serum, 0.5% BSA, and 0.3% Triton X-100, then incubated for 30 minutes with anti-DNP-rabbit polyclonal antibody (1 μg/ml) (Seikagaku Kogyo, Tokyo, Japan) or with IgG from nonimmunized rabbits (1 μg/ml) as a control. They were washed three times in PBS containing 0.3% Triton X-100 for 5 minutes, subsequently reacted with biotinylated goat anti-rabbit IgG (5 μg/ml) in 0.5% BSA in PBS containing 0.3% Triton X-100 for 30 minutes. After washing three times with PBS for 5 minutes, the sections were incubated for 30 minutes according to the ABC method in PBS (diluted 1:50). After washing three times with PBS for 5 minutes, peroxidase activity was visualized by incubating for 2 minutes with 0.1% diaminobenzidine in 50 mM Tris-HCl buffer, pH 7.4, supplemented with 0.02% H₂O₂.

DAMP was detected electron microscopically by the protein A-gold technique on frozen ultrathin sections as described above using anti-DNP antibody. To evaluate the relationship between the pH level and the distribution of cathepsin D in the same phagolysosome and lysosome, double staining was performed. The sections were incubated in 10 μg/ml protein A in 2% gelatin in PBS containing 10 mM glycine before the second step of antibody reaction. The pH was estimated according to the formula pH = 7.0 − log D₁/D₂, where D₁ = density of DAMP-specific gold
particles in the acidic compartment and $D_2 =$ density of gold particles in a neutral compartment (the nucleus). We assumed that the pH in the nucleus was 7.0 according to Orci et al.\textsuperscript{22} and Anderson and Orci.\textsuperscript{19}

**RESULTS**

**Immunogold Localization of Cathepsin D, LGP107, and Opsin During Phagocytic Degradation of Rod Outer Segments**

Figures 1A and 1B show immunogold localization of cathepsin D on the frozen ultrathin sections of the RPE cells, which were fixed 15 minutes after the onset of light. Many phagosomes and phagolysosomes at various stages of degradation of the engulfed ROS were already being seen. These phagosomes and phagolysosomes appeared during the preparation for perfusion-fixation. Cathepsin D was not detected in the majority of phagosomes containing intact ROS. Figure 1A shows a phagosome that appears to be in the early stage of fusion with a lysosome. Many gold particles were seen in the lysosomes, but only a few were seen in the periphery of the ROS in the phagosome. This electron micrograph suggests that cathepsin D was supplied to phagosomes during the fusion with lysosomes. Figure 1B shows a phagolysosome at an advanced stage of degradation. The ultrastructure of the ROS was obscure, and gold particles were distributed evenly on the phagolysosome.

Figure 2 shows localization of LGP107 on the frozen ultrathin sections. LGP107 was detected on both lysosomal and phagolysosomal membranes, but not on the plasma membranes of the RPE cells.

Figure 3 shows immunogold localization of opsin on the RPE section embedded in LR White. The upper phagosome (star) in which each lamellar structure of the ROS was well preserved had many gold particles corresponding to opsin molecules, whereas the phagolysosomes in the lower part (asterisks), in which the lamellar structure of the ROS was obscure, contained only a few gold particles.

To clarify the relationship between the degradation of opsin and the appearance of cathepsin D, the RPE was double labeled with large (8 nm) and small (4 nm) gold particles for opsin and cathepsin D, respectively (Fig. 4). It is evident that large and small gold particles coexist within the same phagolysosomes.

In Figure 5, we plotted the density of large and small gold particles in a single phagosome or phagolysosome.
FIGURE 2. Cryoimmunogold localization of LGP107 in rat RPE cells 15 minutes after the onset of light. Gold particles (4 nm) are observed on the phagolysosomal (asterisk) and lysosomal (stars) membranes. Arrowheads show plasma membrane of the RPE. (Original magnification ×48,000). Bar = 1 μm.

FIGURE 3. Immunogold localization of opsin on the LR White sections. Diameter of gold particles was 8 nm. At 8:45, a phagosome in the upper part of the photograph (star) contains the distinct lamellar structures labeled with a number of gold particles, whereas the phagolysosomes in the middle and lower parts (asterisks) are barely labeled with gold particles. (Original magnification ×25,000.) Bar = 1 μm.

FIGURE 4. Co-localization of cathepsin D (4 nm gold particles) and opsin (8 nm gold particles) using the double labeling immunogold technique on LR White sections. Both sizes of gold particles are co-localized in the same phagolysosomes (asterisks), whereas only small particles are localized in the lysosomes (stars). (Original magnification ×42,500.) Bar = 1 μm.

Light and Electron Microscopic Localization of DAMP and Estimation of pH in the Phagosomes

We detected abundant large gold particles corresponding to opsin on the ROS in the phagosomes, but immunolabeling for opsin rapidly decreased in phagolysosomes in reverse proportion to the increase in the small gold particles corresponding to cathepsin D. The regression line indicating the correlation of the two variables is shown in Figure 5, and the correlation coefficient was calculated to be −0.54.

FIGURE 6A shows light microscopic localization of DAMP in the RPE and the neurosensory retina by using anti-DNP antibody followed by the ABC method. DAMP immunoreactivity was intense in the RPE, and the inner nuclear layer was also reactive. When the sections were incubated with nonimmune rabbit IgG as a control, DAB reactions were barely observed (Fig. 6B).
Acidification of Phagosomes in RPE

**FIGURE 5.** Correlation of the particle density of opsin with that of cathepsin D in the phagolysosomes at 8:15 AM in a representative experiment. Control level of gold labeling, which is measured on mitochondria, is 0.26/μm² (opsin) and 6.39/μm² (cathepsin D), respectively. The line shows regression with a correlation coefficient of −0.54.

DAMP was detected electron microscopically by the immunogold technique. As shown in Figure 7, gold particles were localized in the lysosomes and phagolysosomes. However, they were barely detectable in the ROS, nucleus, endoplasmic reticulum, mitochondria, and the Golgi complexes. No gold particles were detected when DAMP was not injected into the vitreous, or when the sections were incubated with nonimmune rabbit IgG as a control (photographs not shown).

To estimate the relationship between the pH and the distribution of cathepsin D in the same phagolysosome and lysosome, we used a double labeling technique, applying large (8 nm) and small (4 nm) gold particles for anti-DNP antibody and anti-cathepsin D.

**FIGURE 6.** Light microscopic visualization of DAMP in the RPE and neurosensory retina by the ABC method on cryosections. NFL, nerve fiber layer; INL, inner nuclear layer; ONL, outer nuclear layer; IS, inner segment of the photoreceptor; RPE, retinal pigment epithelium. (A) Stained with anti-DNP antibody. (B) Stained with nonimmune IgG. (Original magnification ×256.) Bar = 50 μm.

**FIGURE 7.** Cryoimmunogold localization of DAMP. Diameter of gold particles was 8 nm. Gold particles are localized in the lysosomes (stars) and phagolysosomes (asterisks). (Original magnification ×37,500.) Bar = 1 μm.
antibody, respectively. Figure 8 shows that the large and small particles are co-localized within the same phagolysosome and lysosome. The density of gold particles for DAMP was used to calculate the pH level.\textsuperscript{19,22}

Figure 9 shows the pH levels of phagosomes (phagolysosomes) and lysosomes versus particle density of cathepsin D in the same organelles at 8:15 AM, 15 minutes after the onset of light. Intraphagosomal pH decreased from about 7.0 to 5.2, corresponding to the increase of cathepsin D. A definite correlation was observed between the pH and the particle density of cathepsin D in the phagosomes and phagolysosomes. The average pH of lysosomes was 5.24 ± 0.32.

Ultrastructural Alteration in Bafilomycin-Treated RPE Cells After the Onset of Light

Figure 10A shows bafilomycin-treated RPE cells fixed 2.25 hours after the light onset (at 10:15 AM). Bafilomycin A\textsubscript{1} (10 μM, 10 μl) was injected into the vitreous at 8:00 AM, as described in Methods. A number of large phagosomes were observed in the RPE cells. Figure 10B shows the mock injected control RPE cells at 10:15 AM. The ultrastructure of the mock injected RPE cells was similar to the untreated normal RPE cells. No difference was observed between the bafilomycin-treated and the control (mock-injected) eyes in such organelles as the mitochondria, Golgi complex, endoplasmic reticulum (Figs. 10A, 10B), or in the cells of the inner layers of the retina (data not shown). Similar results were obtained when 10 μl of 100 μM bafilomycin A\textsubscript{1} was injected.

Inhibition of the Acidification of Phagolysosomes by Bafilomycin A\textsubscript{1}

We examined the effect of bafilomycin A\textsubscript{1}, competing weak base chloroquine, and carboxyl ionophore nigericin on the acidification of phagolysosomes using the DAMP procedure. DAMP · 2HCl was administered into the vitreous on the previous day (at 6:00 PM), and these agents were injected again into the vitreous at 8:00 AM. The RPE cells were fixed 2.25 hours after the light onset (at 10:15 AM). As shown in Table 1, 2.25 hours after the onset of light, the difference in pH levels was barely observed between phagolysosomes and lysosomes in the mock-injected (10% DMSO) RPE cells. Bafilomycin A\textsubscript{1} significantly increased intraphagosomal pH, though its effect was slightly weaker than that of chloroquine and nigericin.
Acidification of Phagosomes in RPE

Quantitative Analysis of the Number and Area of the Phagosomes

We examined the changes in the number (Fig. 11A) and area (Fig. 11B) of the phagosomes after the onset of light in the presence or absence of bafilomycin A₁.

Figure 11A shows the number of phagosomes at various times of the light cycle in the bafilomycin-treated and the mock-injected (control) RPE cells. In the mock-injected eyes, the number of phagosomes had already increased by 8:15 AM and peaked at 1.25 hours (9:15 AM) after the onset of light. The phagosomes gradually decreased in number from 10:15 AM to 12:15 PM. In the bafilomycin-treated eyes (100 μM, 10 μl), many phagosomes could be seen in the RPE at 10:15 AM. Between 11:15 AM and 12:15 PM, the number of phagosomes decreased as they did in the mock-injected control eyes.

Figure 11B shows the total area of phagosomes (% of cytoplasm) in the RPE cells. In the mock-injected control eyes, the area of phagosomes peaked at 1.75 hours after the onset of light, and decreased gradually from 10:15 AM to 12:15 PM, whereas in bafilomycin-treated eyes, the area of phagosomes markedly increased at 10:15 AM. This showed that bafilomycin A₁ delayed the degradation of the ROS in the phago-
TABLE 1. Effects of Bafilomycin A₁, Chloroquine, and Nigericin on the Intraphagolysosomal pH

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<th>pH</th>
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<tr>
<td>Lyosomes</td>
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<td>Control (10% DMSO)</td>
<td>4.94 ± 0.15 (n = 16)</td>
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<tr>
<td>Phagolysosomes</td>
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<tr>
<td>Control (10% DMSO)</td>
<td>5.09 ± 0.15 (n = 10)</td>
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<tr>
<td>Bafilomycin A₁ (100 µM)</td>
<td>5.74 ± 0.34* (n = 23)</td>
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<tr>
<td>Chloroquine (10 mM)</td>
<td>6.18 ± 0.30* (n = 37)</td>
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<tr>
<td>Nigericin (50 µg/ml)</td>
<td>6.19 ± 0.25* (n = 12)</td>
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DAMP·2HCl was administered into the vitreous on the previous day (at 6 PM), and three kinds of inhibitors were injected into the vitreous at 8 AM (light onset). The RPE cells were fixed 2.25 hr after the light onset (at 10:15 AM). Frozen ultrathin sections were incubated with anti-DNP antibody and subsequently with protein A gold conjugates. The density of gold particles for DAMP was used to calculate pH. A representative experiment is shown. pH is shown as mean ± SD. Values in parentheses indicate the number of phagosomes or lysosomes analyzed.

* Phagolysosomal pH is significantly higher than that of the control phagolysosomes (P values in unpaired T test < 0.001).

Discussion

The phagolysosomal system in the RPE plays an important role in the physiological functions of the retina. Phagocytosis of the ROS, one of the main functions of the phagolysosomal system, consists of multiple processes such as recognition, binding, ingestion, and degradation. Interruption of any one of these steps leads to disorder of the retina. For instance, insufficiency of digestion leads to the formation of lipofuscin granules.

It has been reported that cathepsin D is abundant in RPE cells. Recently, Bosch et al revealed immunoelectron microscopically that cathepsin D is highly concentrated in the lysosomes and phagolysosomes of the RPE cells and that cathepsin D is supplied to phagosomes by fusion with lysosomes. We have confirmed this observation. It has been suggested that cathepsin D mainly contributes to the degradation of opsin. In this study, we revealed by quantitative analysis that opsin was rapidly degraded in correspondence with the increase in the amount of cathepsin D in the phagolysosomes.

LGP107 was also detected on the lysosomal and phagolysosomal membranes of the RPE cells. LGP107 may be supplied to the phagolysosomal membranes from lysosomal membranes through the fusion of lysosomes with phagosomes. The physiological role of LGP107 has not been clearly defined. This glycoprotein has many sialylated oligosaccharide chains. These highly concentrated oligosaccharide chains are
Acidification of Phagosomes in RPE

It is well known that lysosomes and endosomes are acidic compartments. Phagolysosomes also have been reported to be acidic. We injected DAMP into the vitreous and detected acidic compartments in the retina by light microscopy and acidic organelles in the RPE by electron microscopy. In the RPE cells, DAMP accumulated in phagolysosomes and lysosomes, and we found a definite correlation between the pH and the particle density of cathepsin D. This result suggests that the intraphagosomal pH decreases by fusion with lysosomes.

We found that administration of bafilomycin A₁, a specific inhibitor of V-ATPase, significantly inhibits the acidification of phagolysosomes and results in an increase of the internal pH from 5.09 to 5.74 because of a passive diffusion of protons across the phagolysosomes. However, no further alkalinization was observed. Upon incubation with chloroquine or ionophore, the pH of phagolysosomes increased gradually to pH 6.18–6.19. It is well known that rat liver lysosomes contain large amounts of sialoglycoproteins such as LGP107, which are negatively charged within the lysosomes. It has been suggested by Moriyama et al that a nonproton pump factor such as Donnan equilibrium is involved in maintaining the acidic pH of phagolysosomes.

We also showed that, in spite of the treatment with bafilomycin A₁, the lysosomal enzyme, cathepsin D, was supplied to the phagosome. This result indicates that bafilomycin A₁ does not inhibit the fusion of phagosomes with lysosomes. Opsin in the large phagolysosomes that accumulated in the bafilomycin A₁-treated RPE cells did not lose its immunoreactivity. These results suggest that the degradation of the ROS did not proceed when V-ATPase was inhibited by bafilomycin treatment, although lysosomal enzymes had been supplied to the phagosomes. Therefore, acidification of the phagolysosomes by V-ATPase appeared to be an essential process for the degradation of the contents. This result is consistent with our previous finding, which revealed that bafilomycin A₁ inhibits acidification of lysosomes and degradation of epidermal growth factor in cultured cells without affecting internalization of the protein and its transport to the lysosomes. These results suggest that V-ATPase plays an essential role in both endocytic and phagocytic functions, presumably through its acidifying property.

In this study, it was interesting that 3 to 4 hours after the injection of bafilomycin A₁, the difference in the number (Fig. 11A) and area (Fig. 11B) of phagosomes between the bafilomycin-treated and the control RPE cells disappeared. This result suggests that the effect of bafilomycin A₁ is reversible. Because transvitreal movement of molecules and water is very active, bafilomycin A₁ may be removed rapidly from the vitreous fluid and also from the RPE cells. In the culture cell system, we have shown that the acidity of the lysosome recovered after washing off the drug.

Weak bases such as methylamine and chloroquine are known to increase the intralysosomal pH level.
This increase is believed to be due to the accumulation and protonation of weak bases within the lysosomes rather than the inhibition of V-ATPase. It is known that lysosomes are markedly vacuolated by treatment with these weak bases, and that in experimental chloroquine retinopathy, the RPE cells contained membranous cytoplasmic bodies in their cytoplasm in severe degenerative cases. On the other hand, bafilomycin A₁ did not affect the morphology of cultured cells (including the organelles) when it was used at a concentration of 1 μM. We also found no differences in the ultrastructure of the organelles in the RPE cells between bafilomycin-treated and -untreated eyes. Thus, bafilomycin A₁ could be a useful tool for studying the role of V-ATPase in RPE phagolysosomes.

**Key Words**

retinal pigment epithelium, phagocytosis, acidification, vacuolar-type H⁺-ATPase, bafilomycin A₁

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**References**

23. Orci L, Ravazzola M, Anderson RGW. The condensing vacuole of exocrine cells is more acidic than the mature secretory vesicle. *Nature (Lond).* 1987;326:77–79.
Acidification of Phagosomes in RPE


