Phosphatidic Acid Osmotically Destabilizes Lysosomes through Increased Permeability to K^+ and H^+

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Abstract. Lysosomal destabilization is a critical event not only for the organelle but also for living cells. However, what factors can affect lysosomal stability is not fully studied. In this work, the effects of phosphatidic acid (PA) on the lysosomal integrity were investigated. Through the measurements of lysosomal β-hexosaminidase free activity, intralysosomal pH, leakage of lysosomal protons and lysosomal latency loss in hypotonic sucrose medium, we established that PA could increase the lysosomal permeability to K^+ and H^+, and enhance the lysosomal osmotic sensitivity. Treatment of lysosomes with PA promoted entry of K^+ into the organelle *via* K^+/H^+ exchange, which could produce osmotic stresses and osmotically destabilize the lysosomes. In addition, PA-induced increase in the lysosomal osmotic sensitivity caused the lysosomes to become more liable to destabilization in osmotic shocks. The results suggest that PA may play a role in the lysosomal destabilization.

Key words: Lysosome — Phosphatidic acid — Potassium ion — Proton — Osmotic sensitivity

Introduction

In recent years, interest in the lysosomal destabilization has heightened with the realization that this event is critical not only for the organelle functions but also for cellular viability. The most notable changes in the destabilized lysosomes are leakages of their protons and hydrolases, which cause the organelle to lose their various functions. In addition, leakage of lysosomal enzymes into cytosol can cause necrosis, apoptosis (Brunk et al. 1997) and some pathological changes such as prion encephalopathies (Laszlo et al. 1992), Alzheimer's disease (Nixon et al. 1992), myocardial ischemia (Decker et al. 1980). Previous studies have mostly focused on the effects of lysosomal destabilization. However, factors affecting lysosomal integrity and the mechanisms of lysosomes destabilization are not fully studied.

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Phosphatidic acid (PA) is mainly formed by hydrolysis of phosphatidylcholine. Recent studies demonstrate that PA plays a key role in the regulation of intracellular membrane traffic such as fission of Golgi membranes. This lipid metabolite can induce membrane bending and destabilization (Kooijman et al. 2003). It is of interest to clarify whether PA affects lysosomal membrane properties such as the membrane permeability and the organelle stability. The results of this study show that PA can increase lysosomal membrane permeability to K^+ and H^+, and enhance lysosomal osmotic sensitivity. These membrane changes can destabilize the lysosomes, which provides useful information for the studies of lysosomal pathophysiology.

Materials and Methods

Materials

Carbonyl cyanide m-chlorophenylhydrazone (CCCP), fluorescein isothiocyanate-dextran (FITC-dextran, M_r = 70,000), PA and valinomycin were from Sigma (St. Louis, MO). Percoll was purchased from Amersham (Uppsala, Sweden). Other chemicals used were of analytical grade from Beijing Chimical Factory. All aqueous solutions were prepared with deionized, glass-distilled water.

Preparation of lysosomes

Rat liver lysosomes were isolated by the Percoll gradient centrifugation methods of Jonas with a minor modification to increase lysosomal purity (Jonas et al. 1983). Briefly, rat liver was homogenized in 0.25 mol/l sucrose and centrifuged at 3000 × *g* for 8 min with following incubation of supernatant at 37°C for 5 min in the presence of 1 mmol/l $CaCl_2$ to promote separation of lysosomes from mitochondria (Yamada et al. 1984). Then, the supernatant was centrifuged for 20 min at 20,000 × *g*. The pellet was resuspended in sucrose and mixed with Percoll (2 : 1, by volume), and centrifuged at 40,000 × *g* for 90 min. The dense lysosomal band near the bottom of the gradient was pooled and mixed with 10 volumes of 0.25 mol/l sucrose, and centrifuged at 10,000 × *g* for 13 min to remove Percoll. The purified lysosomes were resuspended in 0.25 mol/l sucrose medium at 2.12 mg protein/ml for use. All performances were carried out at 4°C. Protein was determined according to Lowry et al. (1951).

Assay of lysosomal integrity

Lysosomal integrity was assessed by measuring the activity of lysosomal β-hexosaminidase using 1 mmol/l 4-methylumbelliferyl N-acetyl-β-D-glucosaminide as substrate (Bird et al. 1987). The liberated 4-methylumbelliferone was determined by measuring the fluorescence (excitation, 365 nm; emission, 444 nm) on a Hitachi F-4500 fluorescence spectrophotometer. Activities of the enzyme measured in the absence and presence of 0.36% Triton X-100 designated the free activity and the total activity, respectively. The percentage free activity was calculated as

(free activity/total activity) × 100. Lysosomal enzyme latency can be defined as [1 – (free activity/total activity)] × 100. Loss of lysosomal integrity was determined as increased percentage free activity or loss of lysosomal enzyme latency.

Assay of lysosomal permeability to K^+

The lysosomal permeability to K^+ can be assessed by the osmotic protection method. This approach gives a semiquantitative measure of relative rate of entry of permeant ions (Lloyd and Forster 1986). Lysosome sample (8 μl) was incubated in 120 μl incubation medium (0.125 mol/l K_2SO_4, buffered with 10 mmol/l HEPES/KOH at pH 7.0) in the presence or absence of 15 μmol/l PA at 37°C for the indicated time. Then, a portion of this lysosomal suspension was used for the assay of lysosomal integrity. Increases in the lysosomal permeability to K^+ were determined as increased loss of lysosomal enzyme latency.

Measurement of intralysosomal pH

The intralysosomal pH was measured by the method of Ohkuma et al. (1982). Rat was injected intraperitoneally with FITC-dextran (20 mg FITC-dextran/150 g body weight) and starved for 16 h before decapitation. Lysosomes were prepared as described above. The measurement medium was composed of 0.25 mol/l sucrose or 0.125 mol/l K_2SO_4, buffered at pH 7.0 with 20 mmol/l HEPES/Tris. The fluorescence was measured at two excitation wavelengths (495 and 450 nm) with 510 nm as emission wavelength. Intralysosomal pH was determined from the fluorescence ratio (F_{495}/F_{450}) of the lysosomal sample relative to a standard curve generated as described by Ohkuma and co-workers. All measurements were carried out at 37°C on a Hitachi F-4500 fluorescence spectrophotometer.

Measurement of lysosomal proton leakage

Lysosomal proton leakage can acidify their suspending medium. The acidification of assay medium was measured as described previously (Ohkuma et al. 1982). Briefly, lysosomal samples were added to a 2 ml assay medium (contained 0.25 mol/l sucrose and 0.1 mmol/l p-nitrophenol) at 27 μg protein/ml, followed by the addition of 100 μl 0.75 mol/l K_2SO_4. The absorbance (at 400 nm) of pH sensitive dye, p-nitrophenol, was measured immediately after adding 10 μl 1 mmol/l valinomycin to the medium. The absorbance measurements were continued for 3 min. All measurements were carried out at 25°C on a Unico UV-2100 spectrophotometer.

Assay of lysosomal osmotic sensitivity

Lysosomal osmotic sensitivity was assessed as described previously by examining their integrity after incubation in hypotonic sucrose medium (Wan et al. 2002). First, lysosomal samples (0.45 mg protein/ml) were treated with 3.5 μmol/l PA at 37°C for 10 min. Then, the lysosomal samples were incubated in sucrose medium (concentration as indicated) at 37°C for the indicated time. Then, a portion of this lysosomal suspension was used for the assay of lysosomal integrity. Increases in the free enzyme activity of lysosomes after incubation in hypotonic medium indicate increases in the lysosomal osmotic sensitivity.

Results

Effects of PA on the lysosomal K^+ permeability

The lysosomes *in vivo* are surrounded by 140 mmol/l cytoplasmic K^+. Normal lysosomal membranes show only a limited permeability to K^+, which is favorable for the organelle osmotic stability (Harikumar and Reeves 1983). An increase in the ion permeability can cause influxes of K^+ into the lysosomes and osmotical destabilization. The capability of PA to increase lysosomal K^+ permeability is examined using the osmotic protection method (Lloyd and Forster 1986). As shown in Fig. 1, PA-treated lysosomes in sucrose incubation medium did not increase their free enzyme activity. It indicates that the PA itself did not osmotically destabilize the lysosomes, i.e. PA did not enter the lysosomes. In contrast to the sucrose medium, treating the lysosomes with PA in the K_2SO_4 medium increased their free enzyme activity from 12 to 42%, indicating that PA increased the lysosomal K^+ permeability and caused influx of K^+ into the lysosomes. According to electroneutral principle, entry of K^+ into the lysosomes must be accompanied by another ion to compensate the charges of K^+. It is impossible for K^+ to enter lysosomes without movement of another charge-compensating ion even though the lysosomal K^+ permeability increased. K^+/H^+ exchange is a major pathway for the entry of K^+ into lysosomes (Casey et al. 1978). In order to fully exhibit increased lysosomal K^+ permeability, protonophore CCCP was added to the K_2SO_4 medium to permeabilize lysosomal membrane to protons. Thus, entry of K^+ into the PA-treated lysosomes depends solely on their K^+ permeability. In other words, CCCP elim-

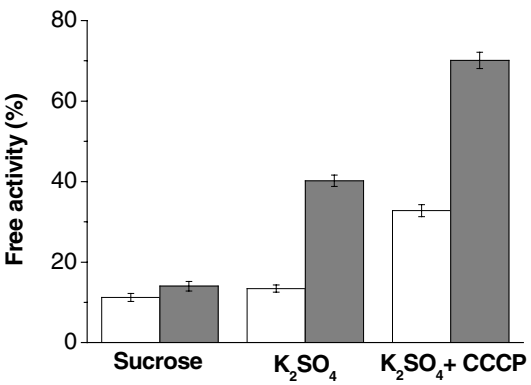

Figure 1. Effects of PA treatment on the lysosomal permeability to K^+. Lysosomal permeability to K^+ was assessed as described in Materials and Methods. Lysosomes were incubated in 0.125 mol/l K_2SO_4 medium or 0.25 mol/l sucrose medium (buffered at pH 7.0 with 10 mmol/l HEPES/KOH) at 37°C for 20 min. Lysosomal free enzyme activity was measured immediately after the incubation. Open bar: lysosomes incubated in the absence of PA; closed bar: lysosomes incubated in the presence of 10 μmol/l PA. 5 μmol/l CCCP was added to the medium as indicated. Values are means ± S.D. of three measurements.

inates electroneutral limitations for the K^+ entry by virtue of K^+/H^+ exchange. The results show that free enzyme activity of the PA-treated lysosomes increased more markedly than the control lysosomes. The marked latency loss of PA-treated lysosomes is due to their increased permeability to K^+.

Effects of PA on the lysosomal H^+ permeability

As demonstrated previously, to what extent K^+ enters lysosomes correlates not only to their K^+ permeability but also to the membrane permeability to H^+ (Casey et al. 1978). Lysosomal membranes show a limited permeability to H^+ (Harikumar and Reeves 1983). Following experiments were performed to examine whether PA could increase lysosomal H^+ permeability. As shown in Fig. 2, treatment of the lysosomes with PA increased their internal pH from 5.55 (line 1, not treated with PA) to 5.63 (line 2) in the sucrose medium and from 5.95 (line 3, not treated with PA) to 6.46 (line 4) in the K_2SO_4 medium. It indicates that the PA treatment increased lysosomal H^+ permeability. The larger pH increase by the PA treatment in K_2SO_4 medium is due to a K^+/H^+ exchange. It is supported by the results that the lysosomal pH increased more markedly upon addition of valinomycin (ionophore of K^+) to the medium (lines 5 and 6).

The intralysosomal acidity is maintained by their H^+-ATPase mediated proton translocation and the limited membrane permeability to H^+ (Harikumar and Reeves 1983). Increases in the lysosomal H^+ permeability should induce an efflux of their protons. Using pH sensitive probe p-nitrophenol, we re-examined whether the PA treatment caused leakage of lysosomal protons. The results show that PA-

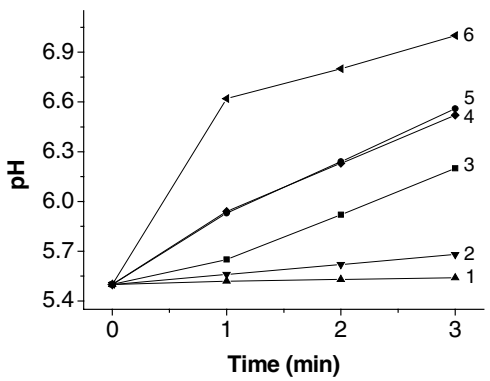

Figure 2. Effects of PA treatment on the intralysosomal pH. Intralysosomal pH was measured at indicated time after lysosomal sample (0.45 mg protein/ml) was added to 1 ml of following measuring medium: 0.25 mol/l sucrose medium (line 1); 0.25 mol/l sucrose medium containing 5 μmol/l PA (line 2); 0.125 mol/l K_2SO_4 medium (line 3); 0.125 mol/l K_2SO_4 medium containing 5 μmol/l PA (line 4); 0.125 mol/l K_2SO_4 medium containing 4 μmol/l valinomycin (line 5); 0.125 mol/l K_2SO_4 medium containing 5 μmol/l PA and 4 μmol/l valinomycin (line 6).

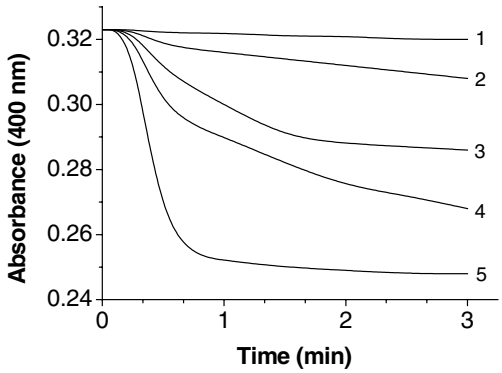

Figure 3. Effects of PA treatment on the lysosomal proton leakage. All assay medium contained 0.25 mol/l sucrose and 0.1 mmol/l p-nitrophenol. Some of the mediums contained additional reagents: 5 μmol/l PA, the medium was buffered at pH 7.0 with 20 mmol/l HEPES/Tris (line 1); control lysosomes (no PA was added, line 2); 2 μmol/l PA (line 3); 5 μmol/l PA (line 4); 5 μmol/l PA and 10 μmol/l CCCP (line 5). Lysosomal samples were added to a 2 ml assay medium at 27 μg protein/ml, followed by the addition of 100 μl 0.75 mol/l K_2SO_4. The absorbance (400 nm) was measured immediately after adding 10 μl 1 mmol/l valinomycin to the medium.

treated lysosomes decreased absorbance (400 nm) of the measuring medium (Fig. 3, compared lines 3 and 4 with line 2). Since unprotonated p-nitrophenol molecules have a sufficiently larger extinction coefficient at 400 nm over that of protonated molecules (Lozier et al. 1976), the absorbance decreases reflect acidifications of the medium. Effects of lysosomal proton leakage on the dye absorbance was further established by the evidence that the absorbance decreased more markedly in the presence of CCCP (line 5) and that decrease in the absorbance could be abolished in the buffered measuring medium (line 1). These results reinforce the above conclusion that the PA treatment increased lysosomal H^+ permeability.

K^+ entered PA-treated lysosomes via K^+/H^+ exchange

The oppositely directed transmembrane concentration gradients of H^+ and K^+ may drive an exchange of lysosomal H^+ for the external K^+. In order to further establish the occurrence of K^+/H^+ exchange, we suspended lysosomes in K^+-containing medium that was buffered at different pH. The resulted different pH gradient across lysosomal membranes could provide different driving force for K^+/H^+ exchange. As shown in Fig. 4, treatment of lysosomes with PA increased their free enzyme activity in K_2SO_4 medium. Extent of the lysosomal latency loss increased with elevating the medium pH from 6.0 to 7.4, reflecting that increased pH gradient promoted entry of K^+ into the lysosomes. It strongly suggests that K^+ entered the PA-treated lysosomes *via* K^+/H^+ exchange.

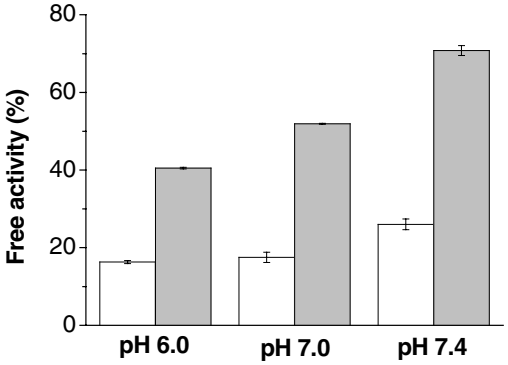

Figure 4. Effects of K_2SO_4 medium pH on the integrity of PA-treated lysosomes. Lysosomes sample (8 μl) was incubated in 120 μl 0.125 mol/l K_2SO_4 medium in the presence or absence of 10 μmol/l PA at 37°C for 30 min. The K_2SO_4 medium was buffered at indicated pH with 10 mmol/l citric acid/HEPES or KOH/HEPES. Lysosomal free enzyme activity was measured immediately after the incubation. Open bar: lysosomes incubated in the absence of PA; closed bar: lysosomes incubated in the presence of PA. Values are means ± S.D. of three measurements.

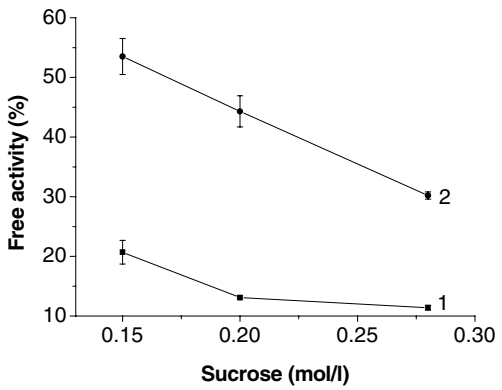

Figure 5. Effects of PA treatment on the lysosomal osmotic sensitivity. Lysosomal sample (0.45 mg protein/ml) was pretreated with 5 μmol/l PA at 37°C for 10 min. Then, the sample was incubated in sucrose medium (concentration as indicated) at 37°C for 20 min. Lysosomal free enzyme activity was measured immediately after the incubation. Line 1: lysosomes were not treated with PA; line 2: lysosomes were treated with 5 μmol/l PA. Values are means ± S.D. of three measurements.

Effects of PA on the lysosomal osmotic sensitivity

Lysosomes are sensitive to osmotic stresses (Lloyd and Forster 1986). An increase in the lysosomal osmotic sensitivity can cause the organelle to become more sus-

ceptible to osmotic shocks, i.e. the lysosomes are prone to osmotic swelling and lysis. As demonstrated above, the PA treatment enhanced lysosomal permeability to K^+ and H^+. Increased uptake of K^+ can cause osmotic imbalance across the lysosomal membranes. Whether the PA-treated lysosomes can resist such osmotic stresses depends largely on their osmotic sensitivity. Lysosomes are named intracellular 'osmometer' because the organelle is sensitive to osmotic shocks (Lloyd and Forster 1986). Increases in their osmotic sensitivity can cause the lysosomes liable to disrupt in osmotic stresses. Effects of the PA treatment on the lysosomal osmotic sensitivity were examined after incubation in hypotonic sucrose medium. The results show that treatment of lysosomes with PA increased their free enzyme activity markedly in the hypotonic sucrose medium (Fig. 5). Compared with control lysosomes (line 1), the PA-treated lysosomes lost their normal ability to resist hypotonic pressures. It suggests that PA increase lysosomal osmotic sensitivity.

Discussion

PA is formed mainly by PLD-mediated hydrolysis of phosphatidylcholine. Various lines of evidence indicate that PA affects a variety of cellular activities including ligand-mediated secretion, cytoskeletal reorganisations, respiratory burst, prostaglandin release, cell migration, cytokine release, and mitogenesis (Steed and Chow 2001). As reported recently, PA plays a role in phagocytosis. The localization of PA in the phagosomal cup and its charged nature may serve to recruit or activate essential components of the fusion machinery. PA is also known to promote phosphatidylinositol 4,5-biphosphate (PI(4,5)P2) synthesis by stimulating type I PI(4,5)P2 kinase. Because PI(4,5)P2 may rapidly and transiently associate with forming phagosomes, it is possible that PA-induced PI(4,5)P2 may act as a lipid anchor for the growing number of phosphoinositide-binding proteins involved in membrane trafficking (Corrotte et al. 2006). In addition, PA is required for the formation of Golgi vesicle and for the transport of vesicles from the endoplasmic reticulum to the Golgi complex (Hong et al. 2001). It was proposed that PA affects local membrane curvature and thus facilitates membrane bending and fission (Kooijman et al. 2005). In addition to these physiological effects, PA can produce pathological effects in living cells. It was reported that the amount of PA is considerably elevated in the pathogenesis of delirium tremens (Rakhimov et al. 1988). Recent study shows that the increase in PA in wild-type plants may be responsible for destabilizing membrane bilayer structure, resulting in a greater propensity toward membrane fusion and cell death (Welti et al. 2002). These evidences suggest that PA plays some roles in these pathological events. Whether PA can produce detrimental effects on the cellular organelles is still unclear. In this study, we established that PA could increase lysosomal osmotic sensitivity and the permeability to K^+ and H^+. As a result, the lysosomes were destabilized. As described above, leakage of lysosomal enzymes can cause necrosis, apoptosis and various diseases. The

lysosomal destabilization induced by PA may correlate to some cell pathological changes.

As shown in this study, PA can increase the permeability of lysosomes to K^+ and H^+, and the lysosomal osmotic sensitivity. It is consistent with previous proposals that PA is a membrane perturbant and that PA can change the biophysical property of membranes (Rizzo et al. 1999). At present, how PA affects membrane permeability is unclear. As proposed previously, solutes permeate membranes either by solubility-diffusion mechanism or through membrane transient defects. To cross membranes by the former mechanism, the permeating ions or molecules must incorporate into the hydrophobic region and then diffuse across the membrane. Since the electrostatic energy of ions is much lower in a water medium with high dielectric constant (∼80) than that of membrane interior with low dielectric constant (∼2), additional energy (Born energy) is required to transfer water or charged particles from the external aqueous phase to the membrane interior (Orme et al. 1988). Obviously, most ions and polar molecules are difficult to dissolve in the hydrophobic region of membrane owing to the Born energy barrier. In other words, it is difficult for these solutes to cross membranes by solubility-diffusion mechanism. The transient defects of membrane are produced by the thermal fluctuation of membrane lipids, which provides another pathway for solute permeation. The defects acting as mobile holes can carry small molecules and ions across membranes (Trauble 1971). Since ions can easily enter the water-filled defects on the membrane surface, the defects save the energy required for the entry of ions into the membrane and facilitate the ion permeation across the membrane. Owing to this reason, transient defects are proposed as the major pathway for ion permeation (Paula et al. 1996). It has been established that fatty acids can readily partition into membranes (Pjura et al. 1984) and that the incorporation of external perturbants can produce transient defects in the membranes (Deamer and Bramhall 1986). It is likely that PA produced defects in the lysosomal membranes and therefore increased the lysosomal permeability to K^+ and H^+.

In the past years, more attentions have been paid to the destabilization of lysosomes due to the serious results of the event. In addition to the induction of necrosis and apoptosis, the leaked lysosomal enzymes can also bring about harmful effects in the pathogenesis of many diseases such as poliovirus infection (Guskey et al. 1970), complement activation-produced lung injury (Hatherill et al. 1989), acute tissue injury (Fell and Dingle 1963) and so on. By now, the factors affecting lysosomal integrity and how these factors destabilize lysosomes are not well studied. According to the obtained evidence, active oxygen (Wan et al. 2002), osmotic stress (Wan et al. 2002), action of phospholipase (Zhao et al. 2005), necrosis and apoptosis (Brunk et al. 1997) can cause lysosomal disintegration. One mechanism for lysosomal destabilization correlates to the damages or alterations in their membranes. It was established that oxidation of lysosomal membrane lipid (Wan et al. 2002) and changes in the membrane physical state can destabilize the lysosomes (Yang et al. 2000). These events can increase lysosomal permeability and membrane fragility, causing destabilization of the lysosomes. As lysosomes are susceptible to osmotic

stress (Lloyd and Forster 1986), the occurrence of osmotic stresses is another cause for lysosomal destabilization. The ability of lysosomes to resist osmotic shocks and the extent of lysosomal osmotic disruption correlates to their osmotic sensitivity and membrane osmotic fragility. PA increased lysosomal permeability to K^+ and H^+, resulting osmotic imbalance across their membranes. PA also increased lysosomal osmotic sensitivity, which caused the lysosomes to become more sensitive to the osmotic shocks. In addition, the incorporation of PA into lysosomal membranes can perturb the membrane lipid order, which may cause changes in their membrane properties and increase the membrane fragility. Thus, the destabilizing effect of PA on the lysosomes may correlate to the above two mechanisms.

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