Lysosome destabilization by cytosolic extracts, putative involvement of Ca^{2+}/phospholipase C

Hui-fang Zhao[b], Xiang Wang[a,c], Guo-Jiang Zhang[a,*]

[a] *Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, PR China*
[b] *University of Science and Technology of Beijing, PR China*
[c] *Graduate School of the Chinese Academy of Sciences, PR China*

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Abstract **Lysosomal disintegration is a crucial event for living cells, but mechanisms for the event are still unclear. In this study, we established that the cytosolic extracts could enhance lysosomal osmotic sensitivity and osmotically destabilize the lysosomes. The cytosol also caused the lysosomes to become more swollen in the hypotonic sucrose medium. The results indicate that the cytosol induced an osmotic shock to the lysosomes and an influx of water into the organelle. Since the effects of cytosol on the lysosomes could be abolished by *O*-tricyclo[5.2.1.0^{2,6}]dec-9-yl dithiocarbonate potassium salt (D609), a specific inhibitor of cytosolic phospholipase C (PLC), the PLC might play an important role in the lysosomal osmotic destabilization. The activity of cytosolic PLC and the extent of enzyme latency loss of the cytosol-treated lysosomes exhibited a similar biphasic dependence on the cytosolic Ca^{2+} concentration. In addition, the cytosol did not osmotically destabilize the lysosomes until the cytosolic calcium ions rose above 100 nM. It suggests that the destabilization effect of cytosol on the lysosomes is Ca^{2+}-dependent.**

Keywords: Lysosome; Phospholipase C; Osmotic sensitivity; Calcium ion

1. Introduction

Lysosomes participate in the physiological turnover of cellular macromolecules such as proteins, lipids, nucleic acids and carbohydrates. The organelle is acidified by its H^+-ATPase, which provides favorable pH conditions for its various acidic hydrolases. To carry out the above functions, the lysosomes must maintain their integrity. The destabilization of lysosomes not only influences their normal activities but also affects cell vitality. Forty years ago, lysosomes were named 'suicide bag' of cells owing to the cytotoxic effects of their hydrolases leaking into cytoplasm [1]. In recent years, interest in the lysosomal destabilization has heightened with the realization that leakage of the lysosomal enzymes, especially cathepsins, can cause necrosis and apoptosis [2,3]. Although, the lysosomal disintegration is a crucial event for living cells, little is known about the mechanisms for the organelle destabilization. To elucidate this issue is very important for the studies of apoptosis, necrosis and lysosomal pathophysiology [4].

The lysosome acts as an intracellular 'osmometer', being susceptible to osmotic destabilization [5]. In the past, a number of studies have focused on the lysosomal osmotic stability. Some investigators paid attentions to the K^+ entry-induced lysosomal osmotic stress and the osmotic protection to lysosomes by their H^+-ATPase-mediated proton translocation [6,7]. The active oxygen-induced oxidation of lysosomal membrane thiol groups can enhance the organelle osmotic sensitivity [8]. In addition, the physical state of lysosomal membranes also influences their osmotic sensitivity [9]. The above studies elucidated some biophysical mechanisms for the lysosomal osmotic destabilization. By now, little information is available concerning the biochemical events that affect the lysosomal osmotic sensitivity. Generally, the cytosolic phospholipase C (PLC) is activated in apoptosis, necrosis and other cellular pathological changes [10–12]. The purpose of this study is to establish if the cytosolic PLC could destabilize the lysosomes and to elucidate the mechanism for the enzyme activation. It may have some significance for the above studies.

2. Materials and methods

2.1. Chemicals

Acridine orange, aprotinin, chymostatin, dibucaine, ethylene glycol-bis-(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), Fluo-3, HEPES, leupeptin, mepacrine, 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide, pepstatin, phenylmethanesulfonyl fluoride, *O*-tricyclo[5.2.1.0^{2,6}]dec-9-yl dithiocarbonate potassium salt (D609) were from Sigma (St. Louis, MO). Amplex Red Phosphatidylcholine-Specific PLC Assay Kit was purchased from Molecular Probes (Eugene, OR). Other analytical grade chemicals used were obtained from Beijing Chemical Factory.

2.2. Preparation of lysosomes

Rat liver lysosomes were isolated by the Percoll gradient centrifugation methods of Jonas et al. [13] with a minor modification to increase lysosomal purity. Briefly, liver cell homogenate was centrifuged for 8 min at $3000 \times g$, the supernatant was incubated at 37 °C for 5 min in the presence of 1 mM $CaCl_2$ to promote separation of lysosomes from mitochondrias [14]. Then, the supernatant was centrifuged for 10 min at $20\,000 \times g$. Following centrifugations were performed as

*Corresponding author.
E-mail address: zhanggj@sun5.ibp.ac.cn (G.-J. Zhang).

Abbreviations: EGTA, ethylene glycol-bis-(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; D609, *O*-tricyclo[5.2.1.0^{2,6}]dec-9-yl dithiocarbonate potassium salt; PLC, phospholipase C

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the methods of Jonas. Purified lysosomes were resuspended in 0.25 M sucrose medium at protein concentration of 2.12 mg/ml for use. Protein was determined according to Lowry et al. [15].

2.3. Preparation of cytosol

Rat liver cytosol was prepared by the methods of Ohkuma and coworkers [16]. Briefly, the liver homogenate was made in 3 volumes of sucrose buffer (10 mM HEPES/KOH, pH 7.0) containing 5 μg/ml leupeptin, pepstatin, chymostatin, aprotinin and phenylmethanesulfonyl fluoride, and centrifuged for 5 min at $3200 \times g$. The resultant supernatant was centrifuged for 10 min at $5280 \times g$. The supernatant obtained was centrifuged further for 70 min at $183\,000 \times g$ using Hitachi CP100MX centrifuge with P70AT rotor. The resulted supernatant, i.e., the cytosol, was immediately frozen in liquid nitrogen and stored at −80 °C until use.

2.4. Measurement of cytosolic Ca^{2+}

The measuring medium contained 100 mM KCl, buffered at pH 7.0 with 10 mM Mops/KOH. Ca^{2+} probe fluo-3 was used at 2 μg/ml to measure cytosolic Ca^{2+}. The fluorescence F, F_{max} and F_{min} were measured (excitation 506 nm, emission 526 nm), respectively, upon sequential additions of 5 μl filtrated cytosol, 2 μl 300 mM $CaCl_2$ and 20 μl 1 M EGTA to the 1 ml measuring medium. Cytosolic $[Ca^{2+}]$ was calculated according to the equation: $[Ca^{2+}] = K_d^*(F - F_{min})/(F_{max} - F)$, K_d = 390 nM (from Handbook of fluorescent probes and research products). Cytosolic $[Ca^{2+}]$ was set to different concentration with EGTA.

2.5. Assay of cytosolic PLC activity

Activity of cytosolic PLC was measured using Amplex Red phosphatidylcholine-specific PLC assay kit. Briefly, 300 μL cytosol containing different calcium concentration was incubated with 2 μL 100 mg/mL lecithin at 37 °C for 90 min. Then, the PLC activity was measured according to the protocol of the assay kit. The cytosol without lecithin was used as negative control. The fluorescence (excitation 563 nm, emission 587 nm) was measured in a fluorescence microplate reader (Thermo, Fluoroskan). Each point was corrected for background fluorescence by subtracting the values derived from the negative control.

2.6. Assay of lysosomal integrity

Lysosomal integrity was assessed as described previously by measuring the activity of lysosomal β-hexosaminidase using 2 mM 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide as substrate [17]. 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 Triton X-100 are designated the free activity and the total activity, respectively. 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 loss of lysosomal enzyme latency or increased percentage free activity.

2.7. Assay of lysosomal osmotic sensitivity

Lysosomal osmotic sensitivity was assessed as described previously by examining their integrity after incubation in hypotonic sucrose medium [8,18]. Briefly, lysosomal samples were incubated in sucrose medium at indicated concentration at 37 °C for the indicated time, then a 60-μl portion of this lysosomal suspension was used for the assay of lysosomal integrity.

2.8. Microscopic observation of lysosomes

To study the effects of cytosol on the lysosomal size, 10 μl lysosomes were treated with either 22.5 μl cytosol or 22.5 μl 0.25 M sucrose (control lysosomes) at 37 °C for 5 min. Meanwhile, the lysosomes were stained with 1 μM fluorescence dye acridine orange. Then, the lysosomal samples were incubated either in 0.15 M sucrose medium or in 0.25 M sucrose medium for 5 min. After the incubations, the lysosomal size was compared between control and cytosol-treated lysosomes using a fluorescence microscope (OLYMPUS IXT1, excitation 590 nm, emission 620 nm).

2.9. Other procedures

2.9.1. Treatment of lysosomes with cytosol. Lysosomes (2.12 mg protein/ml) were treated with cytosol (2.22 mg protein/ml, cytosolic $[Ca^{2+}]$ was adjusted at indicated concentration by the addition of EGTA) at 37 °C for 5 min.

2.9.2. Incubation of lysosomes in sucrose medium. Control lysosomes or cytosol-treated lysosomes were incubated in sucrose medium at indicated concentration at 37 °C for the indicated time. Then, the lysosomal integrity was assessed as described above.

3. Results

The lysosomes are named intracellular 'osmometer' due to their relatively higher sensitivity to osmotic shocks [5]. The osmotic sensitivity of lysosomes reflects the ability of the organelle to resist hypotonic pressures. To assess the lysosomal osmotic sensitivity, we measured the free activity of β-hexosaminidase (a lysosomal marker enzyme) after incubating the lysosomes in a series of hypotonic sucrose medium for a period of time. As shown in Fig. 1, the cytosol-treated lysosomes (line 1) had a greater percentage of free enzyme activity than the control lysosomes (line 2) at each hypotonic concentration of the sucrose medium. It indicates that the cytosol-treated lysosomes were more susceptible to the increased osmotic imbalance across their membranes and therefore lost their enzyme latency markedly. The destabilization effects of the cytosol on the lysosomes were further established by the evidence that the percentage of free enzyme activity of the cytosol-treated lysosomes increased more greatly and rapidly during an incubation in the hypotonic sucrose medium (Fig. 2, line 1) than that of the control lysosomes (line 2). These results indicate that the cytosol caused the lysosomes to lose their normal ability to resist hypotonic pressure and to become more sensitive to an osmotic stress.

Theoretically, an increase in the lysosomal osmotic sensitivity should induce an increased influx of water into the lyso-

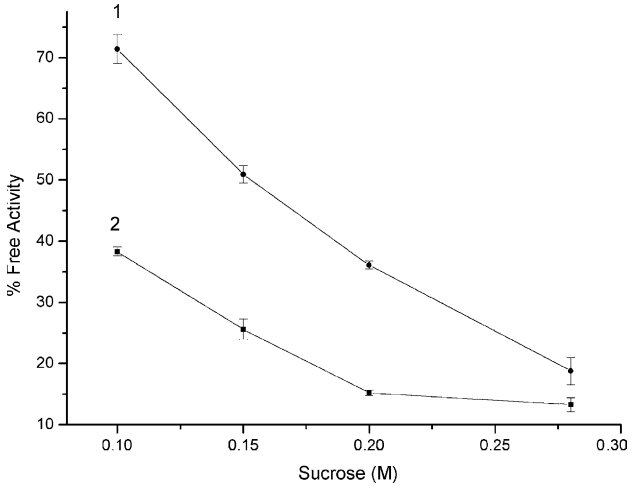

Fig. 1. Effects of sucrose concentration on the osmotic stability of cytosol-treated lysosomes. Cytosolic free calcium ions were adjusted to 316 nM with 0.05 mM EGTA. Lysosomes were treated by the cytosol at 37 °C for 5 min. Then, the lysosomal samples were incubated in the sucrose medium at indicated concentrations for 5 min. Lysosomal free β-hexosaminidase activity was measured immediately after the incubation. Line 1, cytosol-treated lysosomes; line 2, control lysosomes. Detailed procedures were as described in Section 2. Values are means ± S.D. of three measurements.

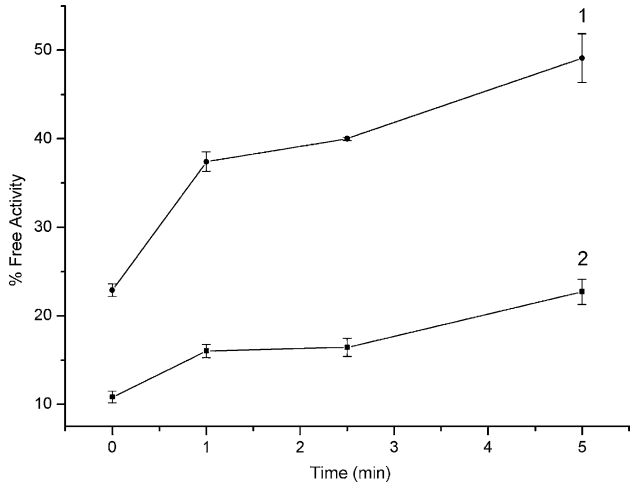

Fig. 2. Effects of incubation duration on the osmotic stability of cytosol-treated lysosomes in hypotonic sucrose medium. Lysosomes were treated by the cytosol as described in Fig. 1 at 37 °C for 5 min. Then, the lysosomal samples were incubated in 0.15 M sucrose medium for the indicated time. Lysosomal free β-hexosaminidase activity was measured immediately after the incubation. Line 1, cytosol-treated lysosomes; line 2, control lysosomes. Detailed procedures were as described in Section 2. Values are means ± S.D. of three measurements.

somes during an osmotic stress. As a result, the organelle should swell to some extent. In order to obtain direct evidence concerning whether the cytosol treatment enhanced the lysosomal osmotic sensitivity, we compared the size of cytosol-treated lysosomes with control ones after a brief incubation in hypotonic sucrose medium. The lysosomes were visualized by the fluorescence of acridine orange using a fluorescence microscope. As shown in Fig. 3, the control lysosomes only slightly swelled during a 5-min incubation in 0.15 M sucrose medium (average lysosomal diameters of Fig. 3A and B are 2.0 and 2.5 μm, respectively). It suggests that the control lysosomes can resist slight osmotic imbalance across their membranes within a short period and that the lysosomes may exhibit only a limited osmotic sensitivity. In contrast to the control ones, the cytosol-treated lysosomes enlarged markedly under the same incubation conditions (average lysosomal diameters of Fig. 3C and D are 2.3 and 4.9 μm, respectively). The result reinforces the above conclusion that the cytosol treatment could enhance the lysosomal osmotic sensitivity. It also further establishes that even a slight osmotic stress could make the cytosol-treated lysosomes swollen. In addition, the results also show that the size of cytosol-treated lysosomes (Fig. 3C) is similar to that of control lysosomes (Fig. 3A) after a 5-min incubation in isotonic sucrose medium. Although, the lysosomal osmotic sensitivity was enhanced by the cytosol treatment, the lysosomes still maintained their normal size under the isotonic condition. The results suggest that the lysosomes with higher osmotic sensitivity were not markedly destabilized until an osmotic stress occurred.

In this work, we first demonstrated the effects of cytosol on the lysosomal osmotic sensitivity. It is of interest to elucidate by what mechanism the cytosol enhanced the lysosomal osmotic sensitivity. The lipid bilayer moiety of biomembranes is considered to be the primary barrier to free diffusion of water and solutes [19]. We have previously demonstrated that the physical state of lysosomal membrane lipids can affect the organelle osmotic sensitivity [9]. It is very likely that the phospholipids of lysosomal membranes play an important role in the regulation of the organelle osmotic property. It has been reported that the cytosolic phospholipase can act on the lysosomal membranes [16]. Enlightened by these evidences, we studied whether the cytosolic phospholipase could affect the lysosomal osmotic sensitivity and which phospholipase played the role. The results show that the latency loss of the cytosol-treated lysosomes in the hypotonic sucrose medium, i.e., the enhancement of the lysosomal osmotic sensitivity, was completely abolished by D609, a specific inhibitor of PLC (Fig. 4). In contrast, mepacrine and dibucaine, the inhibitors of phospholipase A_2, did not prevent the effect of cytosol on the lysosomal osmotic sensitivity. It rules out the role of PLA_2 on the lysosomes and convincingly suggests that the PLC might play a key role in the enhancement of lysosomal osmotic sensitivity.

The above conclusion seems to be contradictory to the evidence that the lysosomes in living cells are stable in the presence of cytosolic PLC. To elucidate under what conditions the cytosolic PLC may osmotically destabilize lysosomes is important for the studies of apoptosis, necrosis and lysosomal pathophysiology. It is well known that Ca^{2+} plays an important role in the regulation of cytosolic PLC activity [20]. In order to answer the above question, we studied the effects of cytosolic Ca^{2+} on the cytosol PLC activity and the influences of Ca^{2+} upon the effects of the PLC on the lysosomal osmotic sensitivity. As shown in Table 1, the PLC activity and the percentage free enzyme activity are relatively low at 50 nM cytosolic Ca^{2+}. The two values increased markedly when the Ca^{2+} rose above 100 nM. Generally, the intracellular calcium ions level at 10–100 nM. The ions can rise above 100 nM during stimulations [21]. The results suggest that the cytosolic PLC exhibits a low activity at normal concentration of the cytosolic Ca^{2+} and therefore the enzyme does not actively react on the phospholipids of lysosomal membranes. However, the Ca^{2+} may activate the cytosolic PLC to some extent when the ions rise above 100 nM. Thus, the enzyme may react on the lysosomal membrane phospholipids, resulting in the enhancement of lysosomal osmotic sensitivity. Interestingly, the PLC activity gradually decreased when the Ca^{2+} concentration rose above several hundred nanomoles. Meanwhile, the enzyme gradually lost its effect on the lysosomal osmotic sensitivity. This result is consistent with previous evidence that the activity of cytosolic PLC exhibits a biphasic dependence on the Ca^{2+} concentration [22].

4. Discussion

Lysosomal integrity is very important not only for the organelle activities but also for the cell vitality. The destabilization of lysosomes can lead to serious results such as leakage of lysosomal protons, loss of their membrane potential and even the disfunction of lysosomes. The most remarkable alteration in destabilized lysosomes is the loss of their enzyme latency. In recent years, a number of studies demonstrated that cathepsins, the lysosomal proteases, could modify Bid (a member of Bcl-2 family) and activate caspase-3 after leaking into cytoplasm, and then induced apoptosis [3]. By what mechanisms the lyso-

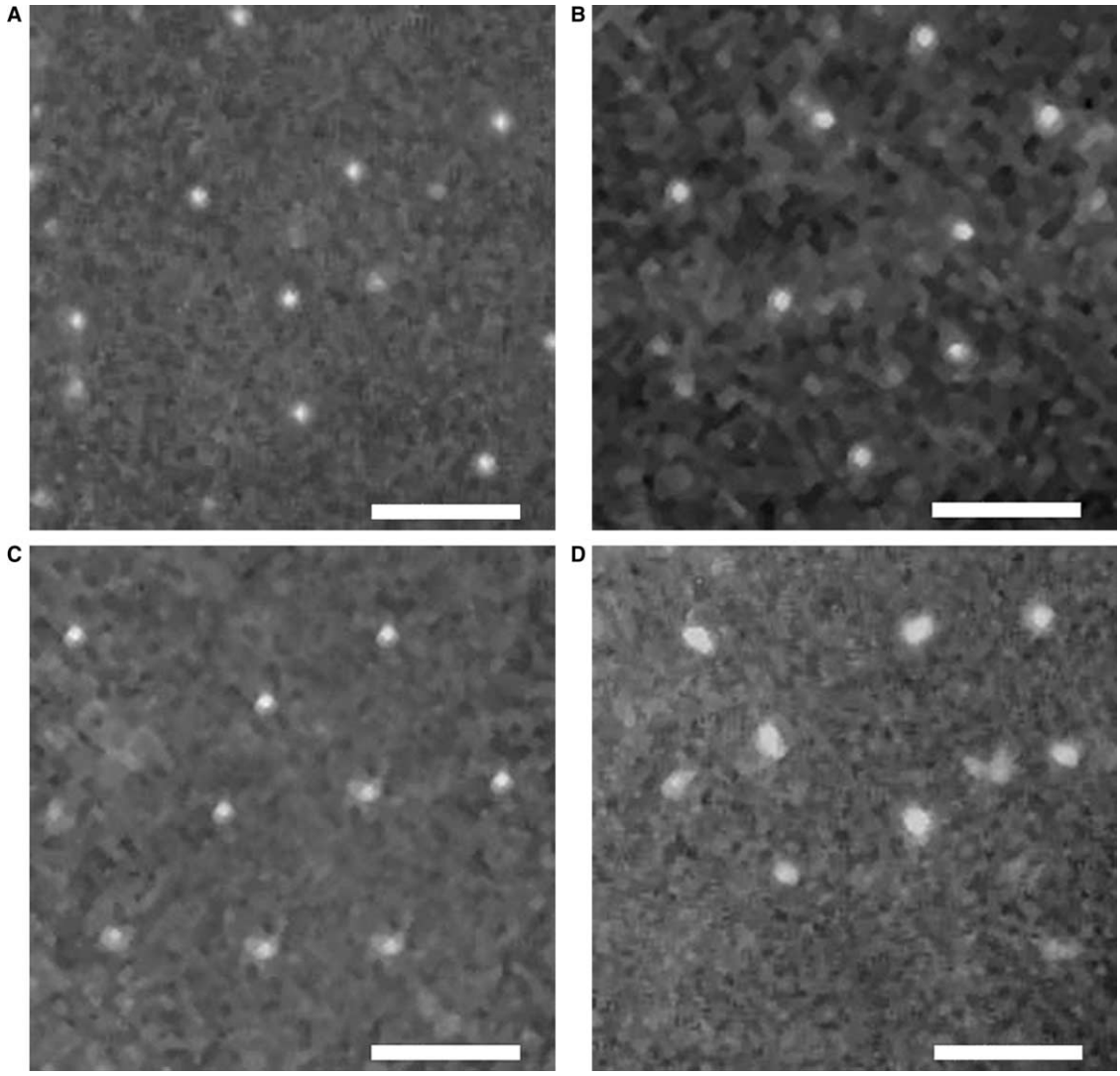

Fig. 3. Effects of cytosol on the lysosomal size. Lysosomes were treated or not with cytosol as described in Fig. 1 at 37 °C for 5 min. Meanwhile, the lysosomes were stained with 1 μM acridine orange. After incubating the lysosomes in 0.15 M sucrose medium or in 0.25 M sucrose medium at 37 °C for 5 min, the lysosomes were observed using fluorescence microscope: (A) control lysosomes incubated in 0.25 M sucrose medium; (B) control lysosomes incubated in 0.15 M sucrose medium; (C) cytosol-treated lysosomes incubated in 0.25 M sucrose medium; (D) cytosol-treated lysosomes incubated in 0.15 M sucrose medium. Detailed procedures were as described in Section 2. A typical result out of three experiments is shown. Bars, 37.5 μm.

somes are destabilized in apoptosis is still unknown. The lysosomal hydrolases can also cause necrosis if the leakage of the enzymes is extensive and serious [2]. The mechanisms for the lysosomal disintegration in the necrosis are also unclear. In the present study, we first established that the cytosolic PLC could destabilize lysosomes through increases in the organelle osmotic sensitivity. It may be of importance for the studies of apoptosis and necrosis.

The osmotic sensitivity of lysosomes is an important property of the organelle. An increase in the lysosomal osmotic sensitivity can cause the organelle to become more susceptible to osmotic shocks, i.e., the lysosomes are prone to osmotic swelling and lysis. In recent years, we established that oxidizing lysosomal membrane thiol groups [8], photooxidizing lysosomal membrane lipids [23] and fluidizing lysosomal membranes [9] could enhance their osmotic sensitivity, which resulted in the destabilization of lysosomes. In addition to these biophysical events, some acidotropic reagents such as methylamine (a weak-base amine) are prone to enter lysosomes. The amine accumulation produces an osmotic imbalance across the membranes and causes an influx of water, resulting in an osmotic shock to the lysosomes [24]. In this study, we established that the cytosolic PLC could increase lysosomal osmotic sensitivity and destabilize the lysosomes in an osmotic stress. The above evidences indicate that the osmotic sensitivity of lysosomes is an important property for the organelle integrity. In living cells, some events may induce an osmotic stress to the lysosomes. The lysosomes of mammalian cells are surrounded by a high concentration of cytoplasmic K^+ (140 mM). The danger of lysosomal osmotic disintegration caused by the entry and accumulation of K^+ in the lysosomes has been emphasized for a long time [6,7]. In addition, the glucagons may produce an osmotic shock to the lysosomes in cellular autophagy [25]. Since the lysosomes are often destabilized in apoptosis and necrosis [2,3] and the cytosol is prone to become hypotonic in the process of cell death [26], we sup-

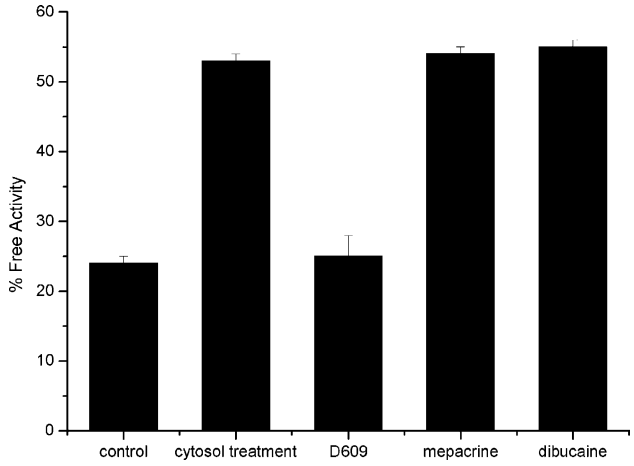

Fig. 4. Effects of cytosolic phospholipase C on the osmotic stability of cytosol-treated lysosomes in hypotonic sucrose medium. Lysosomes were treated by the cytosol as described in Fig. 1 at 37 °C for 5 min either in the absence of any phospholipase inhibitor or in the presence of D609, mepacrine and dibucaine, respectively. Then, the lysosomal samples were incubated in 0.15 M sucrose medium for 5 min. Lysosomal free β-hexosaminidase activity was measured immediately after the incubation. Detailed procedures were as described in Section 2. Values are means ± S.D. of three measurements.

Table 1
Effects of cytosolic calcium ion concentration on the cytosolic PLC activity and lysosomal free enzyme activity

Cytosol [Ca^{2+}] (nM)	PLC activity (fluorescence arbitrary units/h)	Free β-hexosaminidase activity (%)
50	38.8 ± 2.0	10.8 ± 0.3
113	42.5 ± 4.6	27.2 ± 1.8
316	66.7 ± 4.4	41.3 ± 3.1
1830	46.9 ± 3.9	31.8 ± 1.8
18360	26.9 ± 2.1	14.8 ± 1.3

Cytosolic PLC activity at each cytosolic calcium ion concentration was measured as described in Section 2. For measuring free β-hexosaminidase activity, the lysosomes were treated by the cytosol at indicated calcium ion concentration at 37 °C for 10 min, then incubated in 0.25 M sucrose medium at 37 °C for 10 min. Free enzyme activity was measured immediately after the incubation. Values are means ± S.D. of three measurements.

pose that some factors of these events may induce an osmotic stress to the organelle and disintegrate them osmotically. It remains for future studies to determine these factors. In conclusion, the occurrence of an osmotic imbalance across lysosomal membranes may osmotically destabilize the lysosomes, while an increase in their osmotic sensitivity can promote the destabilization. In this respect, the effect of cytosolic PLC on the lysosomes is notable.

Lysosomal osmotic sensitivity was assessed as described previously by examining their integrity after incubation in hypotonic sucrose medium [8,18]. Using this method, we produced a series of osmotic imbalance across the lysosomal membranes and examined the lysosomal ability to resist the hypotonic pressure against their membranes. Compared with control lysosomes, the cytosol-treated lysosomes lost their normal ability to stand hypotonic pressure and were more sensitive to the osmotic stress. This conclusion is supported by additional observations that the cytosol-treated lysosomes markedly swelled in the hypotonic medium. Since the effect of cytosol on the lysosomes could be abolished by D609, it is likely that the PLC might cause the lysosomes to become more susceptible to osmotic destabilizations. It may remind investigators to notice the effects of activated cytosolic PLC on the lysosomal integrity in the studies of apoptosis and necrosis.

In this study, we established that the cytosolic PLC might increase lysosomal osmotic sensitivity and that Ca^{2+} regulated the enzyme activity. It is consistent with previous conclusion that Ca^{2+} is an important regulator of the cytosolic PLC [20]. Lysosomal destabilization is an abnormal change of the organelle. The above results raise two questions: why the lysosomes are stable in the presence of cytosolic PLC in normal case and in what situation the enzyme can osmotically destabilize lysosomes. As demonstrated previously, the activity of cytosolic PLC exhibits a biphasic dependence on the Ca^{2+} concentration (low activity either at normal concentration of cytosolic Ca^{2+} or above 10 μM, the enzyme is markedly activated when the Ca^{2+} rise above 100 nM and gradually lost its activity if the ion concentration is elevated above 1 μM) [22]. This study obtained a similar result. Normal intracellular calcium ions level at 10–100 nM. The ions can rise up to several micromolar during stimulations [21]. As described above, the cytosolic PLC maintains a low activity at normal concentration of cytosolic Ca^{2+}. It is reasonable that the enzyme does not decompose lysosomal membrane phospholipids in normal case. However, the enzyme may break the membrane lipids if it is activated by a rise of cytosolic Ca^{2+}. A number of studies established that the intracellular Ca^{2+} rose markedly in some pathological alterations such as necrosis [27], anoxia-caused cell injury [28] and toxic cell death [29]. On the other hand, the cytosolic PLC is activated in many pathological events [10–12]. As reported recently, the lysosomes are often disintegrated in necrosis and apoptosis [2,3]. However, the mechanisms underlying the events are still unknown. Based on the results of this study, we suppose that the increases in the cytosolic Ca^{2+} in some pathological changes may activate the cytosolic PLC and that the activated PLC might osmotically destabilize the lysosomes.

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