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Mechanism of cytosol phospholipase C and sphingomyelinase-induced lysosome destabilization

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

[a] *Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China*
[b] *Graduate School of the Chinese Academy of Sciences, Beijing 100039, China*
[c] *University of Science and Technology Beijing, Beijing 100083, China*

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Abstract

Lysosomal disintegration may cause apoptosis, necrosis and some diseases. However, mechanisms for these events are still unclear. In this study, we measured lysosomal β-hexosaminidase free activity, membrane potential and intralysosomal pH. The results revealed that the cytosolic extracts of rat hepatocytes could increase the lysosomal permeability to both potassium ions and protons, and osmotically destabilize lysosomes via K^+/H^+ exchange. The effects of cytosol on lysosomes could be completely abolished by D609, which inhibited both phospholipase C and sphingomyelinase, and partly prevented by sphingomyelinase inhibitor Ara-AMP, but not by the inhibitors of PLA_2. Moreover, purified phospholipase C could destabilize the lysosomes while phospholipase A_2 and phospholipase D did not produce such effects. The cytosolic phospholipases hydrolyzed lysosomal membrane phospholipids by 50%, which could be prevented by D609. Disintegration of the cytosol-treated lysosomes biphasically depended on the cytosolic [Ca^{2+}]. The cytosol did not disintegrate lysosomes below 100 nM or above 10 μM cytosolic [Ca^{2+}], but markedly destabilized lysosomes at about 340 nM [Ca^{2+}]. The results suggest that cytosolic phospholipase C and sphingomyelinase may be responsible for the alterations in lysosomal stability by increasing the ion permeability.

Keywords: Lysosome; Phospholipase C; Sphingomyelinase; Calcium ion; Potassium ion; Proton

1. Introduction

Lysosomes participate in the physiological turnover of cellular macromolecules. In normal case, lysosomes maintain their integrity and provide favorable pH conditions for various acidic hydrolases. Destabilization of lysosomes can collapse the pH gradient across their membranes and induce the leakage of various hydrolases, which may in turn cause the loss of lysosomal functions. In addition, lysosomal disintegration is critical to living cells. Since the leakage of lysosomal hydrolases may cause cell death, this organelle was named "suicide bag" of cells [1]. Moreover, the leaked lysosomal enzymes can bring about harmful effects on the pathogenesis of many diseases such as prion encephalopathies [2], Alzheimer's disease [3] and so on. A number of studies demonstrated that leakage of lysosomal enzymes could cause apoptosis and necrosis [4,5]. As pointed out recently by Kroemer, it is very important to elucidate the mechanisms of lysosomal destabilization involved in the cell death [6,7].

Lysosomal membrane phospholipids play important roles in maintaining their integrity. A number of studies demonstrated that cytosolic phospholipases were activated in apoptosis, necrosis and pathogenesis of some diseases [8–10]. Whether cytosolic phospholipases can hydrolyze lysosomal membrane lipids and destabilize lysosomes is unclear. Recently, we established that cytosolic PLC could increase lysosomal osmotic sensitivity [11]. Although injured lysosomes were more liable to disintegrate in hypotonic sucrose mediums, they still

Abbreviations: Ara-AMP, adenine 9-beta-D-arabinofuranoside 5′-monophosphate; [Ca^{2+}], Ca^{2+} concentration; CCCP, carbonyl cyanide mchlorophenylhydrazone; D609, O-tricyclo[5.2.1.02,6]dec-9-yl dithiocarbonate potassium salt; EGTA, ethylene glycol-bis-(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; FITC-dextran, fluorescein isothiocyanate-dextran; oxonol VI, bis(3-propyl-5-oxoisoxazol-4-yl) pentamethine-oxonol; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; SMase, sphingomyelinase.

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

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maintained their integrity in isotonic sucrose medium. It suggests that an osmotic shock is needed to trigger destabilization of lysosomes even though they are injured by phospholipases. The mechanisms how osmotic stresses to the lysosomes are produced remain to be elucidated. The lysosomes of mammalian cells are surrounded by a high concentration of cytoplasmic K^+ (140 mM) [12]. The increase in the lysosomal permeability to K^+ can cause influxes of K^+ into lysosomes, which may produce an osmotic imbalance across the lysosomal membranes and then disintegrate the organelle [13]. Whether cytosolic phospholipases can increase lysosomal K^+ permeability and osmotically destabilize lysosomes is unknown. The purpose of this study is to answer this question and to clarify related mechanisms. It may help to elucidate how lysosomes are disintegrated in apoptosis, necrosis and some diseases, and the mechanisms by which lysosomes induce these cellular changes.

2. Materials and methods

2.1. Materials

Adenine 9-beta-D-arabinofuranoside 5′-monophosphate (Ara-AMP), aprotinin, carbonyl cyanide mchlorophenylhydrazone (CCCP), chymostatin, 2,4′-dibromoacetophenone, dibucaine hydrochloride, Fluo-3, fluorescein isothiocyanate-dextran (FITC-dextran, $M_r = 70{,}000$), leupeptin, 4-methylumbelliferyl N-acetyl-β-D-glucosaminide, O-tricyclo[5.2.1.$0^{2,6}$]dec-9-yl dithiocarbonate potassium salt (D609), pepstatin, 1,10-phenanthroline monohydrate, phenylmethanesulfonyl fluoride, phospholipase A_2 (from honey bee venom), phospholipase C (from *C. welchii*), phospholipase D (from cabbage), quinacrine dihydrochloride and valinomycin were purchased from Sigma (St. Louis, MO). Tributylamine was purchased from Fluka (Buchs, Switzerland). Amplex Red Phosphatidylcholine-Specific Phospholipase C Assay Kit, Amplex Red SMase Assay Kit and bis (3-propyl-5-oxoisoxazol-4-yl) pentamethine-oxonol (oxonol VI) were purchased from Molecular Probes (Eugene, OR). Percoll was purchased from Amersham (Uppsala, Sweden).

2.2. Preparation of lysosomes

Rat liver lysosomes were isolated by the Percoll gradient centrifugation methods of Jonas with a minor modification to increase lysosomal purity [14]. Briefly, rat liver was homogenized in 0.25 M sucrose and centrifuged at 3000 × *g* for 8 min. 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 [15]. 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 vol.), and centrifuged at 40,000 × *g* for 90 min. The lower 1/4 volume of the gradient (lysosomal fraction) was pooled and mixed with 10 volumes of 0.25 M sucrose, and centrifuged at 10,000 × *g* for 13 min to remove Percoll. Purified lysosomes were resuspended in 0.25 M sucrose medium at 2.12 mg protein per ml for use. All performances were carried out at 4 °C. Protein was determined according to Lowry et al. [16] method.

2.3. Preparation of cytosol

Rat liver cytosol was prepared by the methods of Sai et al. [17]. Briefly, liver homogenate was made in 3 volumes of 0.25 M sucrose (10 mM hepes/KOH, pH 7.0) containing 5 μg/ml leupeptin, pepstatin, chymostatin, phenylmethanesulfonyl fluoride and aprotinin, and centrifuged at 3200 × *g* for 5 min using Sigma 3K30 centrifuge. The resulted supernatant was centrifuged at 5280 × *g* for 10 min. Then, the supernatant was centrifuged at 20,000 × *g* for 20 min. Obtained supernatant was centrifuged further at 183,000 × *g* for 70 min at 4 °C using Hitachi CP100MX ultracentrifuge (P70AT rotor). The resulted supernatant, i.e. the cytosol (2.22 mg protein per ml), was frozen immediately in liquid nitrogen and stored at –80 °C for use.

2.4. Assay of lysosomal integrity

Lysosomal integrity was assessed through the measurement of lysosomal β-hexosaminidase activity using 1 mM 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide as substrate [18]. Liberated product 4-methylumbelliferone was determined by measuring its 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% (v/v) Triton X-100 was designated free activity and 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 increased percentage free activity or loss of lysosomal enzyme latency. All chemical reagents and solutions used in this study did not affect lysosomal integrity.

2.5. Measurement of cytosolic [Ca^{2+}]

Two microliters 2 μg/ml Fluo-3 and 5 μl ultrafiltrated cytosol were added sequentially to 1 ml measurement medium (100 mM KCl, 10 mM Mops/KOH, pH 7.0). The fluorescence (excitation 506 nm, emission 526 nm) was measured at 22 °C on a Hitachi F-4500 fluorescence spectrophotometer. Cytosolic [Ca^{2+}] was calculated according to the equations:

$$[Ca^{2+}]_n = Kd \times (F_n - F_{min})/(F_{max} - F_n)$$

$$\text{Cytosolic}[Ca^{2+}] = \{[Ca^{2+}]_2 \times 1005 - [Ca^{2+}]_1 \times 1000\}/5$$

Where F_1 was the fluorescence measured in the absence of cytosol, F_2 was the fluorescence when cytosol was added. F_{max} and F_{min} were obtained upon addition of 2 μl 300 mM $CaCl_2$ and 20 μl 1 M EGTA/3 M Tris, respectively. The K_d for Ca^{2+}/Fluo-3 complex was determined at 383.01 ± 0.89 nM by the method of Minta et al. [19]. Cytosolic [Ca^{2+}] was set to different concentration with EGTA [20].

2.6. Assay of cytosolic phospholipase C and sphingomyelinase activity

Cytosolic PLC or SMase activities was measured by using the Amplex Red phosphatidylcholine-specific PLC or Amplex Red SMase Assay Kits according to the manufacture' instructions. First, PLC or SMase converts the substrate to form phosphocholine. After the action of alkaline phosphatase, which hydrolyzes phosphocholine, choline is oxidized by choline oxidase to betaine and H_2O_2. Finally, H_2O_2, in the presence of horseradish peroxidase, reacts with Amplex Red reagent in a 1:1 stoichiometry, to generate the highly fluorescent product resorufin. Enzyme activity was expressed as fluorescence arbitrary units. Briefly, 50 μl cytosol samples were incubated with 100 μl Amplex Red reagent/HRP/choline oxidase/alkaline phosphatase working solution for 1 hour at 37 °C to generate the fluorescent product. The fluorescence (excitation 544 nm, emission 590 nm) was recorded using a fluorescence microplate reader (Thermo, Flouroskan). Each point was corrected for background fluorescence by subtracting the values derived from the negative control (absence of substrate).

2.7. Assay of residual phosphorus on the lysosomal membrane

The lipids of 1 ml lysosomal sample was extracted using 3 ml chloroform/methanol (2:1, by vol.) [21]. The lower phase was evaporated and digested with 0.5 ml 70% perchloric acid at 190 °C for 120 min. Measurements of phosphorus were carried out as described in [22]. Briefly, 10 μl of digested sample was mixed with assay solution (0.9 ml 2.5 M HCl, 0.9 ml 26 mg/ml sodium molybdate and 0.5 ml water). A color complex was produced upon addition of 50 μl 1.26 mg/ml malachite green to the mixed solution. The absorbance was measured at 650 nm against a mixed blank in a Unico 2100 spectrophotometer. A calibration curve, obtained by this procedure using KH_2PO_4 solutions of known concentrations, was used to determine the amount of inorganic phosphate present in the assay. All performances were carried out at 25 °C.

2.8. 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 [23]. A 30-μl cytosol-treated or control lysosomes were incubated in 120 μl incubation medium (0.125 M K_2SO_4, 30 μg/ml D609, 10 mM hepes/KOH, pH 7.0) at 37 °C for the indicated time. Then, a 60-μl 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.

2.9. Measurement of lysosomal membrane potential

For measuring lysosomal membrane potential, 16.21 μg protein per ml lysosomal sample, 1.5 μM oxonol VI and 2 μM CCCP were added to 1.7 ml assay medium (0.25 M sucrose, pH adjusted to 6.7 with imidazole), respectively [24]. The contents of blank cuvette were same as that of sample cuvette except addition of lysosomes. Three hundred microliters of 0.75 M K_2SO_4 was added to sample and blank cuvettes at indicated times, respectively. Membrane potential was registered by the absorbance difference $\Delta A_{625-587}$. All measurements were performed at 25 °C on a Hitachi U-3200 spectrophotometer.

2.10. Measurement of intralysosomal pH

Intralysosomal pH was measured by the method of Ohkuma et al. [25]. Briefly, rat was injected intraperitoneally with FITC-dextran (20 mg FITC-dextran/150 g body weight) and starved for 16 hours before decapitation. Lysosomes were prepared as described above. Measurement medium was composed of 0.25 M sucrose or 0.125 M K_2SO_4 (both buffered at pH 7.0 with 20 mM 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 lysosomal sample relative to a standard curve generated as described by Ohkuma. All measurements were carried out at 37 °C on a Hitachi F-4500 fluorescence spectrophotometer.

3. Results

3.1. The cytosol disintegrated lysosomes

In order to assess the effects of cytosol on the lysosomal integrity, we examined the latency loss of β-hexosaminidase (lysosomal marker enzyme) after treating the lysosomes with cytosol. As shown in Fig. 1, the treatment of lysosomes with cytosol increased their free enzyme activity markedly (line 1), while control lysosomes maintained their enzyme latency (line

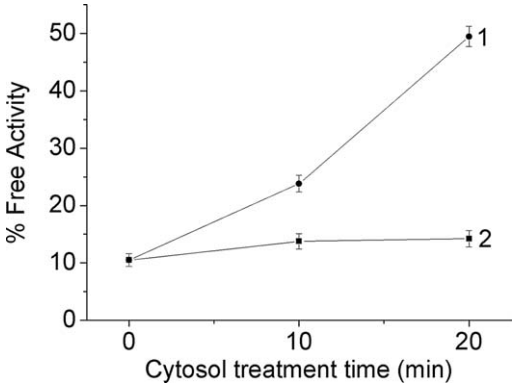

Fig. 1. Effects of cytosol treatment on the lysosomal integrity. Cytosolic free [Ca^{2+}] were adjusted to 341 nM with 0.04 mM EGTA. A 7.5 μl lysosomes were treated with 22.5 μl cytosol at 37 °C for the indicated time. Control lysosomes were not treated by cytosol but also incubated at 37 °C for the indicated time. Lysosomal free β-hexosaminidase activity was measured immediately after the incubation as described in "Section 2". Line 1: cytosol-treated lysosomes, line 2: control lysosomes. Values are mean ± S.D. of three measurements.

2), indicating that the cytosol treatment resulted in the destabilization of lysosomes.

3.2. The cytosol PLC and SMase induced lysosomal destabilization

The lipid portion of lysosomal membranes plays an important role in the maintenance of their integrity. It is of interest to establish whether cytosolic phospholipases could destabilize lysosomes through the hydrolysis of their membrane phospholipids. The results show that the cytosol-induced increase in the lysosomal free enzyme activity was abolished by D609, an inhibitor of PLC [26] (Fig. 2A). Similar results were also obtained by using another PLC inhibitor phenanthroline [27]. Ara-AMP, an inhibitor of SMase [28], decreased the free β-hexosaminidase activity released by 25% when incubated with cytosol. In contrast, dibromoacetophenone, dibucaine and quinacrine, the inhibitors of phospholipase A_2 [29,30], did not bring about any effects. The results suggest that cytosolic PLC played a major role in the lysosomal destabilization and that SMase also produced destabilizing effect on the lysosomes. In an additional experiment, we heated the cytosol to inactivate its enzymes. As a result, the cytosol lost its destabilizing effect on the lysosomes.

The effects of inhibitors on the cytosol PLC and SMase activities were assessed. As shown in Fig. 2B, the cytosol PLC activity was greatly inhibited by D609 and phenanthroline. Recent study reported that D609 is a relative specific inhibitor of PLC owing to its indirect inhibition on SMase [31]. Results (Fig. 2C) illustrated that the cytosol SMase activity could be inhibited not only by Ara-AMP but also by D609. The results might explain why the lysosomes could be protected by D609, phenanthroline and Ara-AMP.

To re-examine whether PLA_2 and PLD affect lysosomal integrity, the following experiments were carried out. As shown in Fig. 3, purified PLA_2 and PLD did not affect lysosomal enzyme latency at 40 mU. The enzymes even at 80 mU were still unable to increase lysosomal free enzyme activities (date not shown). In contrast, the lysosomes could be destabilized by 20 mU purified PLC. Increasing PLC to 40 mU caused a more

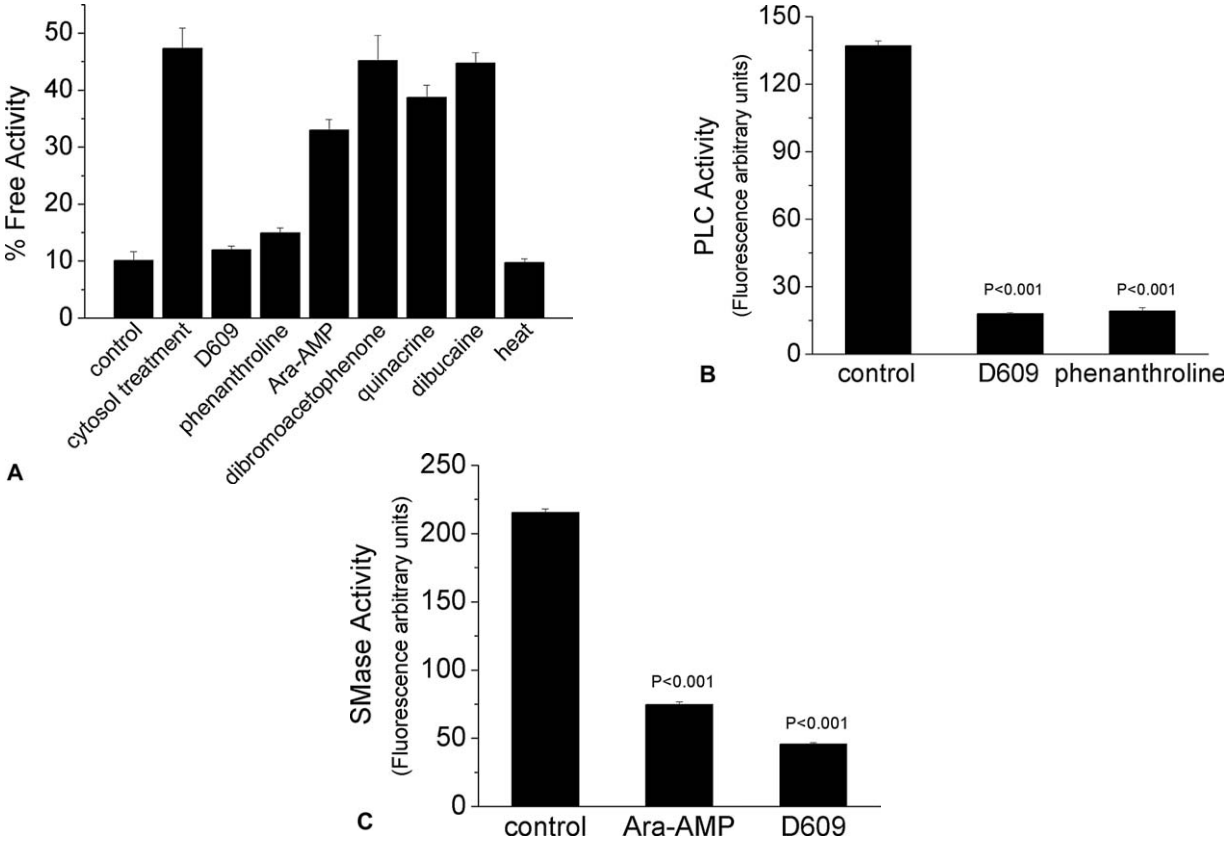

Fig. 2. Effects of cytosolic phospholipases on the lysosomal integrity. A. A 7.5 μl lysosomes were treated with 22.5 μl cytosol in the presence of 30 μg/ml D609, 8.3 mM phenanthroline, 1 mM Ara-AMP, 1 mM dibromoacetophenone, 10 μM quinacrine, and 50 μM dibucaine, respectively, at 37 °C for 20 min. Lysosomal free β-hexosaminidase activity was measured immediately after the incubation. B. Assay of inhibitor effects on the cytosol PLC activity. One hundred and fifty microliters of cytosol was incubated with 1 mM phosphatidylcholine in the absence or presence of 30 μg/ml D609 or 8.3 mM phenanthroline at 37 °C for 2 hours. Then 50 μl of each suspension was used for PLC activity measurement. C. Assay of inhibitor effects on the cytosol SMase activity. One hundred and fifty microliters of cytosol was incubated with 0.5 mM sphingomyelin in the absence or presence of 1 mM Ara-AMP or 30 μg/ml D609 at 37 °C for 2 hours. Then 50 μl of each suspension was used for SMase activity measurement. All cytosol used in A, B and C contained 341 nM free [Ca^{2+}]. Values are mean ± S.D. of three measurements. Statistical analysis was performed using the Student's *t*-test.

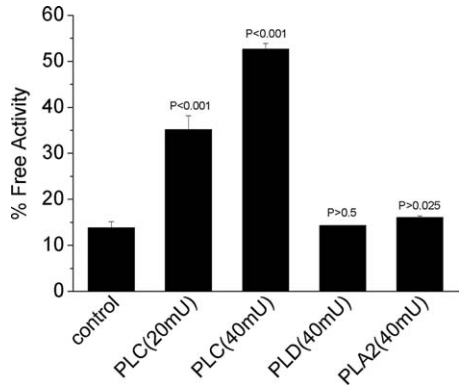

Fig. 3. Effects of phospholipase A_2, C, and D on the lysosomal integrity. 7.5 μl lysosomes were treated with 40 mU/ml phospholipase A_2, 20 and 40 mU/ml phospholipase C, and 40 mU/ml phospholipase D, respectively, at 37 °C for 10 min. Lysosomal free β-hexosaminidase activity was measured immediately after the treatment. Values are mean ± S.D. of three measurements. Statistical analysis was performed using the Student's *t*-test.

marked enzyme latency loss. These findings provided strong support to the above result that PLA_2 and PLD did not affect lysosomal integrity.

Phospholipase C and SMase can hydrolyze membrane phospholipids, resulting in the release of phosphate from the phospholipids. As reported previously, rat liver lysosomal membrane contained PC by 37.7% and spingomyelin by 32.9% of total membrane phospholipids [32]. In order to determine whether the enzymes hydrolyzed lysosomal membrane phospholipids, we measured residual phosphorus of the cytosol-treated lysosomal membranes. As shown in Fig. 4, the treatment of lysosomes with cytosol decreased their membrane phosphorus by 50%. The cytosol-induced loss of membrane phosphorus was almost abolished by D609. Based on the results shown in Fig. 2B,C, the conclusion can be drawn that these two enzymes may break lysosomal membrane structure by hydrolyzing lysosomal membrane phospholipids.

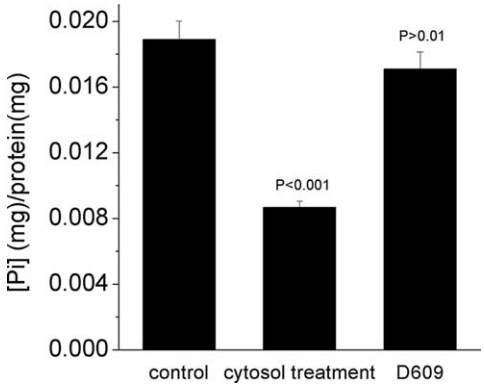

Fig. 4. Hydrolysis of lysosomal membrane phospholipid by cytosolic phospholipases. A 200-μl lysosomal sample was treated with 600 μl cytosol containing 341 nM free $[Ca^{2+}]$ in the absence or presence of 30 μg/ml D609 for 20 min at 37 °C. Residual phosphorus on the lysosomal membrane phospholipids was measured immediately as described in "Section 2". Values are mean ± S.D. of three measurements. Statistical analysis was performed using the Student's *t*-test.

3.3. The hydrolysis of lysosomal membrane phospholipid is $[Ca^{2+}]$ dependent

Both PLC and SMase can hydrolyze membrane phospholipid and generate phosphocholine. To assess the effect of Ca^{2+} on the hydrolysis of lysosomal membrane phospholipid, lysosomal membranes were treated with cytosol in the presence of different $[Ca^{2+}]$. Then, the produced phosphocholine was measured. As shown in Fig. 5A, phosphocholine increased with increasing $[Ca^{2+}]$ from 33 to 340 nM and then decreased when $[Ca^{2+}]$ raised further (up to 19.2 μM). It indicates that Ca^{2+} regulated the phospholipid hydrolysis biphasically. It is similar to the biphasic dependence of PLC activity on $[Ca^{2+}]$ [11]. We also assessed the effects of Ca^{2+} on the activity of cytosol SMase. The results (Fig. 5B) demonstrated that the enzyme activity increased with elevating $[Ca^{2+}]$ up to about 350 nM, similarly to the overall phosphocholine generation curve plotted in Fig. 5A.

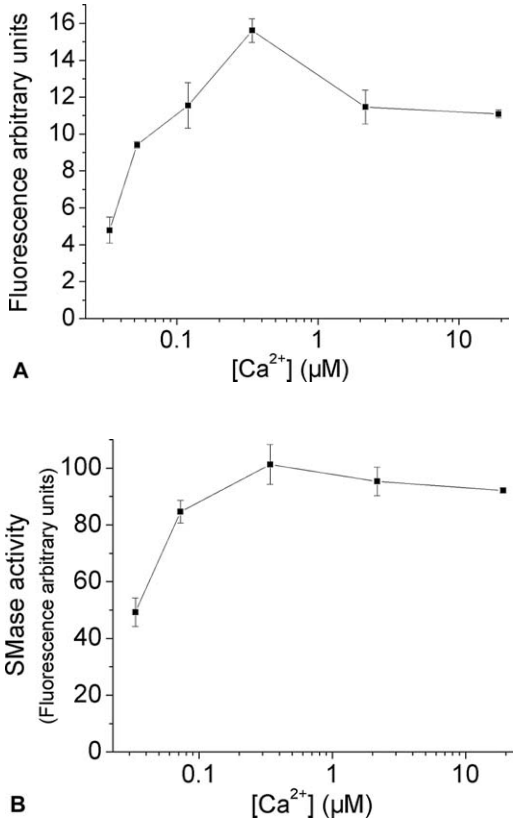

Fig. 5. Production of phosphocholine from cytosol-treated lysosomal membranes is $[Ca^{2+}]$ dependent. A. Three hundred microliters of cytosol containing indicated $[Ca^{2+}]$ was incubated with 2 μl lysosomal membranes (obtained by the centrifugation of 20 μl osmotically disrupted lysosomes) at 37 °C for 90 min. Then, 25 μl suspension was used for the assay of phosphocholine as described in "Section 2". Values are mean ± S.D. of four measurements. B. Effects of cytosolic $[Ca^{2+}]$ on the cytosolic SMase activity. One hundred and fifty microliters of cytosol containing indicated $[Ca^{2+}]$ was incubated with 0.5 mM sphingomyelin at 37 °C for 2 hours. Then 50 μl of each suspension was used for SMase activity measurement. Values are mean ± S.D. of three measurements.

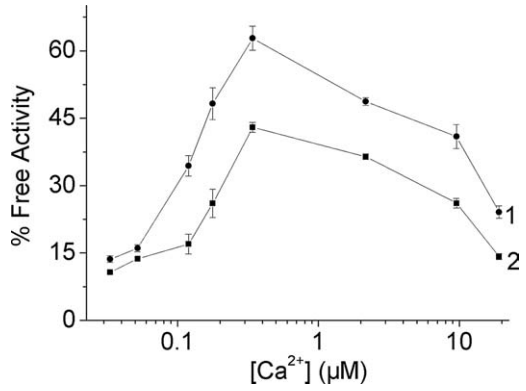

Fig. 6. Influence of cytosolic [Ca^{2+}] on the cytosol-induced lysosomal destabilization. A 7.5-μl lysosomal sample was treated with 22.5 μl cytosol containing indicated [Ca^{2+}] at 37 °C for 10 min. Then, the sample was incubated in 120 μl 0.25 M sucrose medium or 0.125 M K_2SO_4 medium (both contained 30 μg/ml D609 and buffered at pH 7.0 with 10 mM hepes/KOH) at 37 °C for 10 min. Lysosomal free enzyme activity was measured immediately after the incubation. Line 1: incubation in K_2SO_4 medium, line 2: incubation in sucrose medium. Values are mean ± S.D. of three measurements.

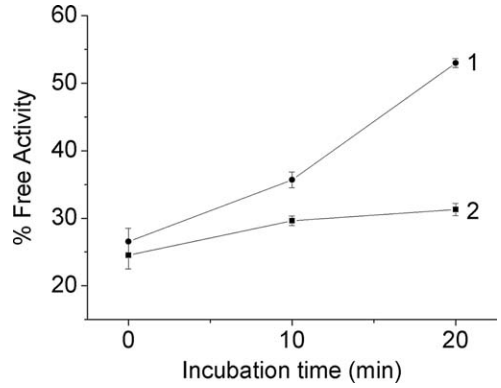

Fig. 7. Cytosol treatment increased lysosomal permeability to K^+. Lysosomal K^+ permeability was assessed as described in "Section 2". A 7.5-μl lysosomal sample was treated with 22.5 μl cytosol containing 341 nM free [Ca^{2+}] at 37 °C for 10 min. Then, the sample was incubated in 120 μl 0.125 M K_2SO_4 medium (containing 30 μg/ml D609 and buffered at pH 7.0 with 10 mM hepes/KOH) at 37 °C for the indicated time. Lysosomal free enzyme activity was measured immediately after the incubation. Line 1: incubation in K_2SO_4 medium, line 2: incubation in sucrose medium. Values are mean ± S.D. of three measurements.

3.4. The cytosol destabilized lysosomes in K_2SO_4 medium

The cytosol of mammalian cells contains abundant potassium ions. Increases in the lysosomal permeability to K^+ can cause influxes of K^+ into lysosomes. The K^+-entry produced osmotic imbalance across lysosomal membranes may osmotically destabilize the lysosomes [33]. To examine the effect of K^+ on the lysosomal integrity, lysosomes were treated with cytosol at different [Ca^{2+}] and then incubated in the K^+-containing medium and sucrose medium, respectively. As shown in Fig. 6, the cytosol-treated lysosomes lost their latency more greatly in the isotonic K_2SO_4 medium than that in the isotonic sucrose medium (compared line 1 with line 2). It suggests that the lysosomes were prone to lose their integrity in the potassium salt medium. The latency loss of cytosol-treated lysosomes showed biphasic dependence on [Ca^{2+}], presumably due to the effect of Ca^{2+} on the cytosolic phospholipases.

3.5. The cytosol increased lysosomal permeability to K^+

The cytosol destabilized lysosomes in K_2SO_4 medium. Since normal lysosomes exhibit only a limited permeability toward K^+ [33], we examined whether the cytosol increased lysosomal K^+ permeability and therefore osmotically destabilized the lysosomes. As shown in Fig. 7, the cytosol-treated lysosomes lost enzyme latency markedly in the K_2SO_4 medium (line 1). In contrast, the lysosomes maintained their integrity in sucrose medium (line 2). Additional measurements show that control lysosomes could maintain their enzyme latency in the K_2SO_4 medium during a 20-min incubation (data not shown). The results indicate that the cytosol treatment increased the lysosomal permeability to K^+. The latency loss of the cytosol-treated lysosomes in K_2SO_4 medium (right end point of line 1, free enzyme activity 53%) could be mostly abolished by D609 (free enzyme activity decreased from 53 to 33%, approximate to the value (27%) of left end point of line 1), suggesting that cytosolic PLC and SMase played a role in the increase of lysosomal K^+ permeability.

The lysosomal permeability to K^+ can also be assessed by the measurement of membrane potential using oxonol VI as a probe. An increase in the differential absorbance ($\Delta A_{625-587}$) of the dye, indicating a more positive interior potential of the membrane, will be observed when K^+ are allowed to enter the lysosomes [24]. As demonstrated previously, the ΔpH across lysosomal membrane produces an internal negative membrane potential and an increase in the lysosomal H^+ permeability can cause the internal membrane potential to become more negative [33]. Since the cytosol treatment may increase lysosomal H^+ permeability, to examine the effects of cytosol on the lysosomal K^+ permeability by the measurement of membrane potential, protonophore CCCP was used to permeablize lysosomes to protons. Thus, the membrane potential of differently treated lysosomes will change only with the alternations of their K^+ permeability. As shown in Fig. 8, differential absorbance of the dye increased upon addition of K^+ to the medium. Addition of valinomycin to permeablize lysosomal membranes to K^+ produced the largest increase in the absorbance (line 1). The increase in differential absorbance of cytosol-treated lysosomes is greater than that of the control lysosomes (compare line 2 with line 3). The absorbance of the former (line 2) approached to that of the valinomycin-permeablized lysosomes (line 1) more closely than that of the latter (line 3). It indicates that the cytosol treatment increased the lysosomal permeability to K^+. The absorbance of cytosol-treated lysosomes increased further upon addition of valinomycin to the medium (line 2) and approached the absorbance of the valinomycin-permeablized lysosomes more closely. It suggests that the permeability to K^+ of the former approximated to that of the latter. The increase in the absorbance of cytosol-treated lysosomes could be abolished by a pretreatment of D609 (line 4), indicating that cytosolic PLC and SMase caused an increase in the lysosomal K^+ permeability.

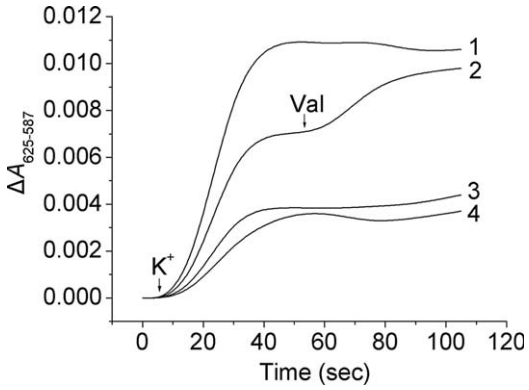

Fig. 8. Effects of cytosol treatment on the lysosomal membrane potential. A 15-μl lysosomal sample was treated with 45 μl cytosol containing 120 nM free [Ca^{2+}] at 37 °C for 10 min. Then, lysosomal membrane potential was measured immediately as described in "Section 2". Line 1: control lysosomes, measuring medium contained 1 μM valinomycin; line 2: cytosol-treated lysosomes, 1 μM valinomycin was added at indicated time; line 3: control lysosomes; line 4: lysosomes were treated with cytosol in the presence of 30 μg/ml D609. A typical result from three experiments is shown.

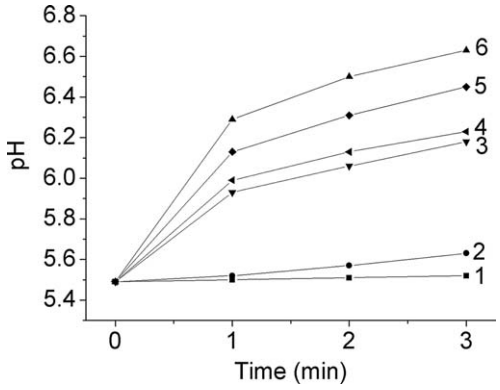

Fig. 9. Effects of cytosol treatment on the intralysosomal pH. All of the cytosol used for the treatment of lysosomes contained 341 nM free [Ca^{2+}]. Line 1: control lysosomes incubated in sucrose medium; line 2: lysosomes treated with cytosol for 4 min and incubated in sucrose medium; line 3: lysosomes treated with cytosol contained 30 μg/ml D609 for 4 min and incubated in 0.125 M K_2SO_4 medium; line 4: control lysosomes incubated in 0.125 M K_2SO_4 medium; line 5: lysosomes treated with cytosol for 4 min and incubated in 0.125 M K_2SO_4 medium; line 6: control lysosomes incubated in 0.125 M K_2SO_4 medium containing 4 μM valinomycin. Intralysosomal pH was measured as described in "Section 2". Values are means of three measurements.

3.6. The cytosol increased lysosomal permeability to H^+

The lysosomes exhibit only a limited permeability toward H^+, which is favorable for the maintenance of their acidification [33]. The oppositely directed transmembrane concentration gradients of H^+ and K^+ may drive an exchange of lysosomal H^+ for the external K^+, which may elevate intralysosomal pH and osmotically destabilize lysosomes. Apparently, an increase in the lysosomal H^+ permeability is detrimental not only to their acidification but also to their stability. Because the cytosol increases lysosomal K^+ permeability, we investigated whether the cytosol could also increase lysosomal H^+ permeability and if K^+ entered cytosol-treated lysosomes through K^+/H^+ exchange. As shown in Fig. 9, the internal pH of control lysosomes is at pH 5.52 in the sucrose medium (line 1) and at pH 6.23 in the K_2SO_4 medium (line 4), respectively, 3-min after incubation. The higher pH value of lysosomes in the K_2SO_4 medium is presumably due to an exchange of external K^+ for the intralysosomal H^+. Treating lysosomes with cytosol elevated their pH from 5.52 (line 1) to 5.63 (line 2) in the sucrose medium and from 6.23 (line 4) to 6.45 (line 5) in the K_2SO_4 medium. It indicates that the cytosol treatment increased the lysosomal permeability to H^+. The larger elevation of intralysosomal pH in the K_2SO_4 medium (increased from 6.23 to 6.45) was caused by a greater efflux of lysosomal protons, presumably to be due to an enhanced K^+/H^+ exchange. The smaller increase in lysosomal pH (increased from 5.52 to 5.63) in sucrose medium was possibly caused by an electroneutral co-migration of lysosomal H^+ with the anions (such as chloride or phosphate) therein. The internal pH of control lysosomes increased greatly up to 6.63 upon addition of valinomycin to the K_2SO_4 medium (line 6), indicating the occurrence of K^+/H^+ exchange. When lysosomes were treated with cytosol in the presence of D609, their internal pH maintained at 6.18 (line 3, similar to the pH 6.23 of control lysosomes) but not rose to 6.45 in the K_2SO_4 medium. It thus suggests that the PLC and SMase may increase the lysosomal permeability to H^+.

3.7. K^+/H^+ exchange of cytosol-treated lysosomes

Passive diffusion of K^+ into lysosomes must be accompanied by the charge-compensating ions to maintain the electroneutrality of the ion movements. It was proved that K^+/H^+ exchange is a major pathway for the lysosomal uptake of K^+. Additional experiments were performed to re-examine whether external K^+ entered the cytosol-treated lysosomes through a K^+/H^+ exchange. The extent of lysosomal uptake of K^+ was assessed by enzyme latency loss. The level of the K^+/H^+ exchange was estimated by the magnitude of K^+-induced elevation of intralysosomal pH. As shown in Table 1, treating the lysosomes with cytosol raised the internal pH from 5.49 of the control lysosomes (in sucrose medium) to 5.94 of the treated lysosomes (in sucrose medium) and 6.92 (in K_2SO_4 medium), respectively. Correspondingly, free enzyme activity of the lysosomes increased from 10.1% (control lysosomes in sucrose medium) to 19.2% (cytosol-treated lysosomes in sucrose medium) and 56.9% (cytosol-treated lysosomes in K_2SO_4 medium), respectively. The greater latency loss (56.9%) and larger increase in the internal pH of the cytosol-treated lysosomes (increased from 5.49 to 6.92) occurred in K_2SO_4 medium but not in sucrose medium. The results indicated that either the latency loss or the elevation of lysosomal pH was dependent on the entry of K^+. The correlation between the lysosomal uptake of K^+ and the elevation of their internal pH suggests that uptake of K^+ by the cytosol-treated lysosomes is via a K^+/H^+ exchange.

Since lowering lysosomal ΔpH can diminish the driving force for K^+/H^+ exchange, we used lysosomotropic weak amine tributylamine to elevate the intralysosomal pH and examined whether the entry of K^+ into cytosol-treated lysosomes

Table 1
Cytosol treatment raised intralysosomal pH and disintegrated lysosomes via K^+/H^+ exchange. Lysosomes were treated or pretreated with 2 mM tributylamine for 5 min. For the cytosol treatment, a 7.5-μl lysosomal sample was treated with 22.5 μl cytosol containing 341 nM free [Ca^{2+}] for 5 min at 37 °C. Then, the lysosomes were incubated either in 0.25 M sucrose medium or in 0.125 M K_2SO_4 for 10 min at 37 °C. Measurements of intralysosomal pH and free enzyme activity were as described in "Section 2". Statistical analysis was performed using the Student's *t*-test

Treatment of lysosomes	Intralysosomal pH, $N = 3$	% Free activity	
		Mean ± S.D., $N = 3$	P values
Control lysosomes[a]	5.49	10.1 ± 0.1	–
Lysosomes treated with tributylamine[b]	5.78	12.8 ± 2.5	$P > 0.1$*
Lysosomes treated with cytosol, then incubated in sucrose medium	5.94	19.2 ± 0.1	–
Lysosomes treated with cytosol, then incubated in K_2SO_4 medium [c]	6.92	56.9 ± 0.4	$P < 0.001$†
Lysosomes pretreated with tributylamine and then treated with cytosol, then incubated in K_2SO_4 medium[d]	6.88	28.0 ± 3.4	$P < 0.001$‡

* b vs. a; † c vs. a; ‡ d vs. c.

was inhibited. As shown in Table 1, the treatment of normal lysosomes with tributylamine increased their pH from 5.49 to 5.78 (in sucrose medium), while the lysosomal latency was not affected (only 12.8%). Treating the lysosomes with tributylamine before the cytosol treatment greatly decreased their free enzyme activity from 56.9% (no tributylamine treatment) to 28.0%. The tributylamine treatment elevated intralysosomal pH and protected the lysosomes from K^+-induced latency loss. It is presumable due to a reduction in K^+/H^+ exchange.

4. Discussion

Lysosome is an organelle responsible for the degradation of cellular macromolecules and endocytic materials. Disintegration of lysosomes can cause the loss of lysosomal functions. Generally, destabilized lysosomes lose their membrane ΔpH, membrane potential and enzyme latency. It is well established that lysosomal membrane ΔpH and membrane potential are important for the across-membrane transportations of many molecules and ions [34,35]. Losses of membrane ΔpH and membrane potential can affect lysosomal transport. An elevation of intralysosomal pH is unfavorable for the activities of acidic hydrolases therein. The inactivation of lysosomal enzymes may cause a variety of serious consequences. The destabilized lysosomes often manifest enhanced membrane permeability, which in turn may promote the influx of external ions and molecules, resulting in osmotic stresses to the lysosomes. Forty years ago, lysosomes were named "suicide bag" of cells owing to the cytotoxic effects of their hydrolases leaking into cytoplasm. Recent studies demonstrated that cathepsins, a family of lysosomal proteases, could modify Bid (a member of Bcl-2 family) and activated caspase-3 after leaking into cytoplasm, and then induced apoptosis [5]. It indicates that disintegration of lysosomes is critical for living cells.

Generally, lysosomes can be destabilized by two kinds of mechanisms. The first one attributes lysosomal destabilization to various damages or alterations in their membranes. In this respect, the destabilizing effects of membrane lipid peroxidation, heavy metal ions (such as lead, copper and iron) and lysosomotropic detergents have extensively been studied by Brunk's group [36–38]. Interestingly, fat-soluble vitamins were found to decrease lysosomal stability [39]. The hydrolysis of lysosomal membrane lipids by their internal phospholipases might destabilize isolated lysosomes under some special conditions [40]. A recent study reported that PLA_2 could destabilize lysosomes in J774 and AG-1518 cells [41]. It is different from our results possibly due to following reasons: First, the authors performed the experiments in J774 and AG-1518 cells, while we treated lysosomes with rat liver cytosol. Second, the destabilization of lysosomes by 0.2 U/ml purified PLA_2 needed a long time treatment (over 1 hour) in their study, while we treated lysosomes with less PLA_2 (40 mU/ml) for a short time (20 min). Owning to these different experimental conditions, we cannot exclude the destabilizing effects of PLA_2 on lysosomes although we did not find such effects of the enzyme.

Another mechanism for the lysosomal destabilization is correlated to the occurrence of osmotic stresses. The lysosome acts as an intracellular "osmometer", being susceptible to osmotic destabilization [23]. An osmotic imbalance across lysosomal membranes can destabilize the lysosomes. As demonstrated previously, changes in the physical states of lysosomal membranes and photooxidation of lysosomal membrane thiol groups can increase the lysosomal osmotic sensitivity and destabilize the lysosomes in osmotic shocks [42,43]. Some molecules such as glucagons may produce osmotic shocks to the lysosomes in cellular autophagy [44]. In addition, the cytosol of apoptotic cells is prone to become hypotonic owing to the efflux of cytoplasmic K^+ [45]. Whether such hypotonic cytosol in apoptosis can destabilize lysosomes is unknown. In a recent study, we established that cytosolic PLC could increase lysosomal osmotic sensitivity [11]. It suggests that the lysosomal membranes are relatively permeable to water and become more fragile in osmotic stresses. Such osmotic-sensitive lysosomes are stable under isotonic conditions but liable to disintegrate in osmotic shocks. Whether the PLC-injured lysosomes disintegrate depends greatly on the occurrence of osmotic stresses. By now, what biochemical events can produce osmotic stresses to lysosomes is not clarified. In this study, we revealed that cytosolic PLC and SMase could produce osmotic shocks to lysosomes via increased permeability to K^+.

The cytosol-induced destabilization of lysosomes might be attributed to the destruction of their membrane lipid structures and the increases in their permeability to K^+ and H^+. The lysosomes in vivo are surrounded by a high concentration of K^+. The K^+-entry induced lysosomal osmotic disintegration was emphasized previously [13]. In normal case, lysosomal membranes exhibit only a limited permeability toward K^+. It is favorable for the lysosomal osmotic stability. However, some

biophysical factors such as low temperature and membrane rigidification can increase lysosomal K^+ permeability [23,24]. In this study, we first established that cytosolic PLC and SMase could also increase lysosomal K^+ permeability.

PLC and SMase can hydrolyze membrane phospholipids, resulting in the release of phosphate from the phospholipids. At present, the mechanism by which the enzymes increase membrane ion permeability is unknown. The transient defects arose from thermal fluctuation in the membrane lipids is proposed as a major pathway for the movements of water and ions across membranes [46]. Perhaps the defects produced by PLC or SMase at the polar head of membrane phospholipids may facilitate the penetration of water into the membranes, which promotes ion diffusion across the membranes. In addition, the enzymes might cause the defective membranes to become fragile. Thus, the lysosomes are prone to disruption.

Lysosomal destabilization is an abnormal change. It should be discussed: why the lysosomes are stable in the presence of cytosolic phospholipases in living cells and in what situation the enzymes can destabilize lysosomes. As shown above, the cytosol-induced lysosomal disintegration correlates to $[Ca^{2+}]$. Normal intracellular calcium ions level at 10–100 nM. Our recent study shows that cytosolic PLC exhibits low activities at such concentrations of cytosolic Ca^{2+} and high activities above 100 nM $[Ca^{2+}]$ [11]. Interestingly, cytosol SMase activity exhibits a similar correlation with $[Ca^{2+}]$ (Fig. 5B). Thus, both of the enzymes do not decompose lysosomal membrane phospholipids under normal conditions but hydrolyze the membrane lipids at 340 nM $[Ca^{2+}]$. By now, there is no evidence showing the dependence of SMase activity on Ca^{2+}. However, several studies have linked PC-PLC-generated DAG to the activation of sphingomyelinase [47–49]. These evidences raise a possibility that Ca^{2+} activates SMase indirectly via the activation of PLC. A number of studies established that intracellular $[Ca^{2+}]$ rose markedly in some pathological alterations such as necrosis, apoptosis, anoxia-caused cell injury, toxic cell death and so on [50,51]. Whether cytosol PLC and SMase can be activated in these pathological events and break lysosomal membrane lipids remains for further study.

Based on the results of this study, we suppose that the increases of cytosolic $[Ca^{2+}]$ in some pathological changes may cause lysosomal destabilization by the action of PLC and SMase. Further studies in living cells are required to verify our hypothesis.

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