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Guanosine 5′-[γ-thio]triphosphate-Mediated Activation of Cytosol Phospholipase C Caused Lysosomal Destabilization

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Abstract. Lysosomal disintegration is critical for the organelle functions and cellular viability. In this study, we established that guanosine 5′-[γ-thio]triphosphate (GTP-γ-S)-activated cytosol of rat hepatocytes could increase lysosomal permeability to both potassium ions and protons and osmotically destabilize the lysosomes via K^+/H^+ exchange. These results were obtained through measurements of lysosomal β-hexosaminidase-free activity, membrane potential and intralysosomal pH. Assays of phospholipase C (PLC) activity show that cytosolic PLC was activated upon addition of GTP-γ-S to the cytosol. The effects of cytosol on the lysosomes could be abolished by D609, an inhibitor of PLC, but not by the inhibitors of phospholipase A_2. The cytosol-treated lysosomes disintegrated markedly in hypotonic sucrose medium, reflecting that the lysosomal osmotic sensitivity increased. Microscopic observations showed that the lysosomes became more swollen in hypotonic sucrose medium. This indicates that the cytosol treatment induced osmotic shock to the lysosomes and an influx of water into the organelle.

Key words: Lysosome — GTP-γ-S — Phospholipase C — Ion permeability — Osmotic sensitivity

Introduction

Lysosomal disintegration is a critical event for the organelle. It brings about various alterations in the lysosomes. Leakage of protons from destabilized lysosomes can raise intralysosomal pH and diminish membrane potential, which decreases the activity of lysosomal acidic hydrolases and inhibits transportation across the membranes of some molecules (Pisoni & Thoene, 1991). These changes can cause loss of lysosomal functions and some metabolic diseases. In addition, lysosomal disintegration may seriously affect living cells. Leakage of lysosomal hydrolases into cytoplasm can cause necrosis and apoptosis (Brunk et al., 1997; Monney et al., 1998). The leaked enzymes from destabilized lysosomes may also induce some diseases such as Alzheimer's disease (Nixon et al., 1992), myocardial ischemia (Decker, Poole & Wildenthal, 1980) and so on. In the past years, effects of lysosomal destabilization on the organelle and living cells were extensively studied, but mechanisms for lysosomal disintegration were not fully elucidated. To clarify this issue is important for the study of lysosome pathology, cell death and the pathogenesis of some diseases (Ferri & Kroemer, 2001).

Lysosomal membrane contains abundant phospholipids, which play important roles in the maintenance of lysosomal integrity (Bode, Baumann & Kinne, 1976). Some studies have demonstrated that photooxidation of lysosomal membrane lipids and rigidification of the membrane may destabilize the lysosomes (Zhang & Yao, 1997; Zhong et al., 2000). These data raise the possibility that changes in lysosomal membrane lipids may destabilize the organelle. A great number of studies have established that cytosolic phospholipases are markedly activated under various pathological conditions, such as necrosis, apoptosis and the pathogenesis of some diseases (Li, Maher & Schubert, 1998; Schluter et al., 1998; Liu et al., 2000). Whether the enzymes can destabilize lysosomes is not well studied. Recently, we established that cytosolic phospholipase C (PLC) could increase lysosomal osmotic sensitivity after activating the enzyme by Ca^{2+} concentration ($[Ca^{2+}]$) at about 340 nM (Zhao, Wang & Zhang, 2005). Generally, intracellular calcium ions level at 10–100 nM (Bootman & Berridge, 1995). Whether cytosolic phospholipases can disintegrate lysosomes at normal cytosolic $[Ca^{2+}]$ is

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unknown. In this study, we established that cytosolic PLC could destabilize lysosomes at normal [Ca^{2+}] when cytosol was activated by guanosine 5′-[γ-thio]triphosphate (GTP-γ-S), providing new information for the mechanism of lysosomal destabilization.

Materials and Methods

Chemicals

Acridine orange, aprotinin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), *O*-tricyclo[5.2.1.02,6]dec-9-yl dithiocarbonate potassium salt (D609), 2,4′-dibromoacetophenone, dibucaine hydrochloride, fluorescein isothiocyanate (FITC)-dextran (M_r = 70,000), GTP-γ-S, leupeptin, 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide, pepstatin, phenylmethyl sulfonyl fluoride (PMSF) and quinacrine dihydrochloride were purchased from Sigma (St. Louis, MO). The Amplex Red Phosphatidylcholine-Specific Phospholipase C Assay Kit and 3,3′-dipropylthiadicarbocyanine iodide ($DiSC_3[5]$) were obtained from Molecular Probes (Eugene, OR). Percoll was purchased from Amersham (Uppsala, Sweden).

Preparation of Lysosomes

Rat liver lysosomes were isolated by the Percoll gradient centrifugation methods of Jonas et al. (1983) with a minor modification to increase lysosomal purity. Rat liver was homogenized in 0.25 M sucrose and centrifuged at 3,000 × *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 mitochondria (Yamada, Hayashi & Natori, 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 vol.) and centrifuged at 40,000 × *g* for 90 min. The lower one-third 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. The purified lysosomes were resuspended in 0.25 M 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).

Preparation of Cytosol

Rat liver cytosol was prepared by the methods of Zhao et al. (2005). The liver homogenate was made in three volumes of 0.25 M sucrose buffer (10 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES]/potassium hydroxide (KOH), pH 7.0) containing 5 μg/ml leupeptin, pepstatin, chymostatin and PMSF and centrifuged at 3,200 × *g* for 5 min using Sigma 3K30 centrifuge. The resulting supernatant was centrifuged at 5,280 × *g* for 10 min. Then, the supernatant was centrifuged at 20,000 × *g* for 20 min. The supernatant obtained was centrifuged further at 183,000 × *g* for 70 min at 4°C using a Hitachi (Tokyo, Japan) CP100MX ultracentrifuge (P70AT rotor). The resultant supernatant, i.e., the cytosol (2.1 mg/ml), was frozen immediately in liquid nitrogen and stored at −80°C for use. Free [Ca^{2+}] of all cytosol used for the treatment of lysosomes was adjusted at 18 nM with ethyleneglycoltetraacetic acid (EGTA).

Assay of Lysosomal Integrity

Lysosomal integrity was assessed by measuring the activity of the lysosomal marker enzyme β-hexosaminidase using 1 mM 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide as substrate (Bird, Forster & Lloyd, 1987). The liberated 4-methylumbelliferone was determined by measuring fluorescence (excitation 365 nm, emission 444 nm) on a Hitachi F-4500 fluorescence spectrophotometer. Activity 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.

Assay of Cytosolic PLC Activity

Cytosolic PLC activity was measured according to the protocol of the Amplex Red Phosphatidylcholine-specific PLC Assay kit. Briefly, 300 μl of cytosol (activated with 500 μM GTP-γ-S or not) was incubated with 2 μl 100 mg/ml of lecithin at 37°C for 90 min. Then, 25 μl of the incubated suspension was used for assay. Fluorescence (excitation 544 nm, emission 590 nm) was recorded immediately after the reaction using a fluorescence microplate reader (Thermo Flouroskan, Vantaa, Finland). Each point was corrected for background fluorescence by subtracting the values derived from the negative control. Activity of the enzyme was expressed as fluorescence arbitrary units per hour.

Assay of Lysosomal Permeability to K^+

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 (Casey, Hollemans & Tager, 1978; Lloyd & Forster, 1986). Lysosomes treated with 37.5 μl cytosol and control lysosomes were incubated in 120 μl of 0.125 M K_2SO_4 (buffered at pH 7.0 with 10 mM HEPES/KOH) 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 lysosomal permeability to K^+ were determined by increased lysosomal enzyme free activity.

Measurement of Intralysosomal pH

Intralysosomal pH was measured by the method of Ohkuma, Moriyama and Takano (1982). Rats were injected intraperitoneally with FITC-dextran (20 mg/150 g body weight) and starved for 16 h before decapitation. FITC-dextran-loaded lysosomes were isolated as described above. Fluorescence measurement medium was composed of 0.25 M sucrose or 0.125 M K_2SO_4, buffered at pH 7.0 with 20 mM HEPES/Tris. Fluorescence was measured at two excitation wavelengths (495 nm and 450 nm), with 510 nm as the 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 et al. All measurements were carried out at 37°C on a Hitachi F-4500 fluorescence spectrophotometer.

Measurement of Lysosomal Membrane Potential

The recording of membrane diffusion potential provides a means of probing the proton permeability of some membranes (Meissner, 1988). The proton permeability of lysosomes can be assessed by measuring their membrane potential using carbocyanine dye $DiSC_3(5)$ as a probe (Harikumar & Reeves, 1983). The assay medium contained 0.25 M sucrose and 0.5 μM $DiSC_3(5)$, buffered at pH 7.0 with 20 mM HEPES/Tris. A 2.5-μl lysosomal sample was used for the assay. Fluorescence measurements were carried out at 25°C, with excitation and emission wavelengths at 622 and 670 nm, respectively, on a Hitachi F-4500 fluorescence spectrophotometer.

Assay of Lysosomal Osmotic Sensitivity

Lysosomal osmotic sensitivity was assessed by examining integrity after incubation in hypotonic sucrose medium (Zhao et al., 2005). Briefly, lysosomal samples were incubated in sucrose medium at the indicated concentrations 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 free enzyme activity of lysosomes after incubation in hypotonic medium indicate increases in lysosomal osmotic sensitivity.

Microscopic Observation of Lysosomes

To study the effects of cytosol treatment on lysosomal size, 7.5 μl of lysosomes were treated with 34 μl of cytosol (activated with 500 μM GTP-γ-S or not) or 34 μl of 0.25 M sucrose (control lysosomes) at 37°C for 5 min. Meanwhile, lysosomes were stained with 7 μM of the fluorescence dye acridine orange. Then, lysosomal samples were suspended in 0.15 or 0.25 M sucrose medium for 10 min. Lysosomes were observed using a fluorescence microscope (Olympus, Tokyo, Japan; IX 71, excitation 470 nm, emission 620 nm).

Results

Effect of GTP-γ-S on Cytosolic PLC-Induced Lysosomal Disintegration

First, we studied the effects of GTP-γ-S-activated cytosol on lysosomal integrity. As shown in Figure 1, free enzyme activities of control and cytosol-treated (without GTP-γ-S) lysosomes were at 18% and 23%, respectively, after a 20-min incubation. This indicates that the cytosol treatment itself did not disintegrate the lysosomes. In contrast, treating lysosomes with cytosol in the presence of GTP-γ-S increased free enzyme activity markedly to 53%, suggesting that cytosolic small G protein played a role in the lysosomal destabilization. In addition, GTP-γ-S could not destabilize lysosomes by itself (*data not shown*). The free activity increase of lysosomes induced by the GTP-γ-S-activated cytosol could be abolished by D609, an inhibitor of PLC (Müller-Decker, 1989; Schütze et al., 1992). However, phospholipase A_2 inhibitors such as dibromoacetophenone, quinacrine and dibucaine (Volwerk, Pieterson & de Haas, 1974; McHowat & Creer, 1998) did not bring about any effect. Knowing that GTP-γ-S-activated cytosol could disintegrate lysosomes, we examined whether cytosolic PLC could be activated under these conditions. The results show that activity of the enzyme increased by 5.7-fold upon addition of GTP-γ-S to the cytosol (cytosolic $[Ca^{2+}]$ at 18 nM). Concretely, PLC activity was 30.11 ± 0.84 and 172.14 ± 1.14 fluorescence arbitrary units/h for control cytosol and GTP-γ-S (500 μM)-activated cytosol, respectively (enzyme activity was measured as described in Materials and Methods). As reported recently, D609 is a relatively specific inhibitor of PLC. It can also indirectly inhibit sphingomyelinase (Suematsu et al., 2003). To clarify whether sphingomyelinase also contributed to the destabilization of lysosomes, we examined the effect of GTP-γ-S on cytosolic sphingomyelinase activity. Addition of GTP-γ-S (500 μM) to the cytosol did not activate sphingomyelinase (*data not shown*). Thus, this rules out a role of sphingomyelinase in lysosomal destabilization and suggests that cytosolic PLC plays a major role.

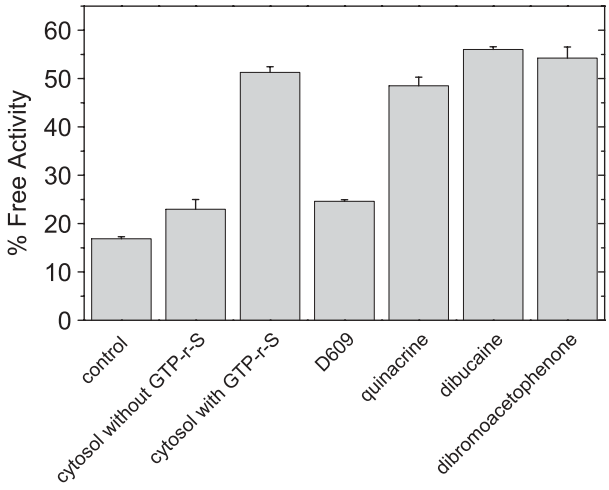

Fig. 1. Effects of cytosolic phospholipase on lysosomal integrity. Lysosomes, 7.5 μl, were treated with 30 μl cytosol containing 500 μM GTP-γ-S or not in the presence of 30 μg/ml D609, 50 μM dibucaine, 10 μM quinacrine and 1 mM dibromoacetophenone, respectively, at 37°C for 20 min. Control lysosomes were not treated with cytosol. Free activity of lysosomal β-hexosaminidase was measured immediately after incubation. Values are means ± standard deviation of three measurements.

Cytosolic PLC Increased Lysosomal Permeability to K^+

In mammalian cells, lysosomes are surrounded by a high concentration of cytoplasmic K^+ (140 mM). Normal lysosomal membranes show only limited permeability toward K^+. An increase in ion permeability can cause influxes of K^+ into the lysosomes, which may produce osmotic imbalance across their membranes and osmotically destabilize the lysosomes (Harikumar & Reeves, 1983). Whether the cytosol treatment increased lysosomal K^+ permeability was examined using the osmotic protection method (Casey et al., 1978; Lloyd & Forster, 1986). As shown in Figure 2, treatment of lysosomes with GTP-γ-S-activated cytosol increased free enzyme activity by 31% during 20-min incubation in K_2SO_4 medium (line 1), while both the cytosol (without GTP-γ-S)-treated (line 2) and control (line 3) lysosomes increased their free enzyme activity by only about 9%. The results indicate that treatment of lysosomes with GTP-γ-S-activated cytosol increased K^+ permeability. The increase in enzyme free activity of the former lysosomes (line 1) could be reduced from 53% to 33%

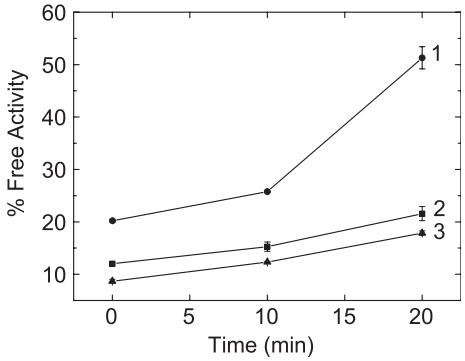

Fig. 2. Effects of cytosol and GTP-γ-S on lysosomal K^+ permeability. Lysosomal samples, 7.5 μl, were treated with 30 μl cytosol in the presence of 500 μM GTP-γ-S or not at 37°C for 10 min. Then, samples were incubated in 0.125 M K_2SO_4 medium (buffered at pH 7.0 with 10 mM HEPES/KOH) at 37°C for the indicated time. Lysosomal free enzyme activity was measured immediately after incubation. Lysosomal K^+ permeability was assessed as described in Materials and Methods: *1*, lysosomes treated with cytosol in the presence of GTP-γ-S; *2*, lysosomes treated with cytosol in the absence of GTP-γ-S; *3*, control lysosomes, not treated with cytosol. Values are means ± standard deviation of three measurements.

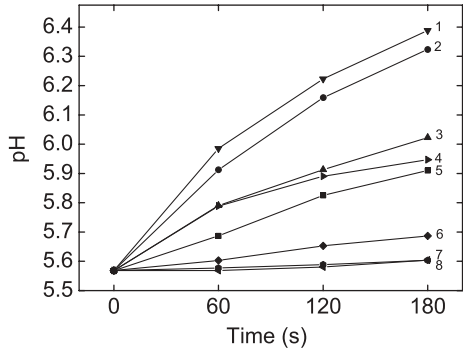

Fig. 3. Effects of cytosol treatment on intralysosomal pH. All measuring media were buffered at pH 7.0 with 20 mM HEPES/Tris. Intralysosomal pH was measured at the indicated time after the sample was added to the medium. Lines *1–5* were measured in 0.125 M K_2SO_4 medium and lines *6–8* were measured in 0.25 M sucrose medium. *1*, Control lysosomes, measuring medium contained 4 μM valinomycin; *2*, lysosomes treated with cytosol in the presence of 500 μM GTP-γ-S for 5 min; *3*, lysosomes treated with cytosol in the presence of 500 μM GTP-γ-S and 30 μg/ml D609 for 5 min; *4*, lysosomes treated with cytosol for 5 min; *5*, control lysosomes; *6*, lysosomes treated with cytosol in the presence of 500 μM GTP-γ-S for 5 min; *7*, lysosomes treated with cytosol for 5 min; *8*, control lysosomes. Values are means of three measurements. Detailed procedures were as described in Materials and Methods.

by pretreatment of the cytosol with D609 (*data not shown*). This suggests that cytosolic PLC plays a role in the increase in lysosomal K^+ permeability.

Cytosolic PLC Increased Lysosomal Permeability to H^+

The influx of K^+ into lysosomes must be accompanied by another charge-compensating ion to maintain the electroneutrality of the ion movements. The oppositely directed transmembrane concentration gradients of H^+ and K^+ may drive an exchange of lysosomal H^+ for the external K^+. Normal lysosomes exhibit only a limited permeability toward H^+ (Harikumar & Reeves, 1983). Apparently, increases in lysosomal H^+ permeability are favorable for K^+/H^+ exchange. The following experiments were designed to examine whether GTP-γ-S-activated cytosol can increase lysosomal H^+ permeability and promote influx of K^+ into the lysosomes through K^+/H^+ exchange. As shown in Figure 3, the internal pH of control (line 8) and cytosol-treated (lines 7 and 6, in the absence and presence of GTP-γ-S, respectively) lysosomes were 5.60, 5.60 and 5.68 in sucrose medium, respectively, after 3-min measurement. Elevation of intralysosomal pH is due to increased efflux of lysosomal H^+. It suggests that lysosomal H^+ permeability increased. When samples were measured in K_2SO_4 medium, internal pH was 5.91, 5.94 and 6.32 (lines 5, 4 and 2), respectively. The higher pH value of each type of lysosomes in K_2SO_4 medium is presumably due to exchanges of external K^+ for intralysosomal H^+. Treatment of lysosomes with GTP-γ-S-activated cytosol caused the largest elevation of internal pH (from 5.68 in sucrose to 6.32 in K_2SO_4), reflecting that exchange of lysosomal H^+ for external K^+ was increased by the treatment. It can be explained by the above results that the cytosol treatment (in the presence of GTP-γ-S) increased lysosomal permeability to both H^+ and K^+. K^+/H^+ exchange across the lysosomal membrane is further verified by evidence that the internal pH of control lysosomes increased greatly up to 6.38 upon addition of valinomycin (K^+-selective ionophore) to K_2SO_4 medium (line 1). Finally, we examined whether cytosolic PLC affected lysosomal H^+ permeability. The larger pH elevation of lysosomes induced by GTP-γ-S-activated cytosol was reduced markedly to 6.02 upon addition of D609 (line 3). This suggests that PLC may increase lysosomal permeability to H^+.

Lysosomal H^+ permeability can also be semiquantitatively assessed by measurement of membrane potential using the carbocyanine dye $DiSC_3(5)$ as a probe (Harikumar & Reeves, 1983). This positively charged dye accumulates within the lysosome that is electrically negative inside with respect to the outside, leading to quenching of the dye fluorescence. An increase in the fluorescence quenching of the dye, indicating a more negative interior of the lysosomes, will be observed when the lysosomal proton permeability increases. As shown in Figure 4a, the magnitude of fluorescence quenching of the cytosol-treated (in the presence of GTP-γ-S, line 3) lysosomes is larger than that of the cytosol-treated (without GTP-γ-S, line 2) and control lysosomes (line 1). This

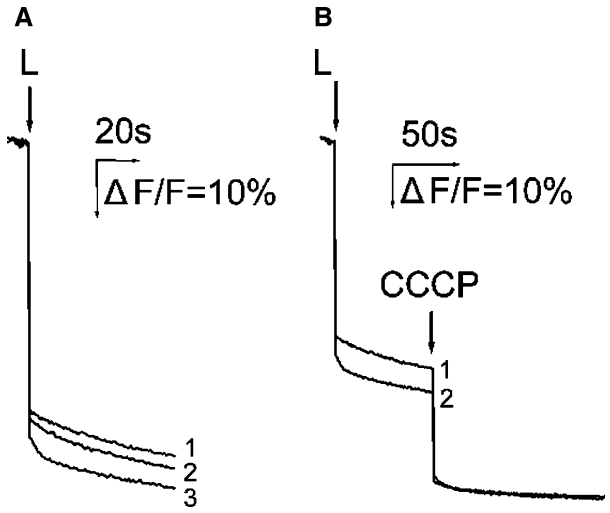

Fig. 4. Effects of cytosol treatment on lysosomal H^+ permeability. Assay medium contained 0.25 M sucrose and 0.5 μM $DiSC_3(5)$, buffered at pH 7.0 with 20 mM HEPES/Tris. Lysosomal sample (L) and CCCP were added to the assay medium at the indicated time. Fluorescence intensity is expressed as a percentage of intensity just before addition of lysosomes (ΔF/F). (*A*) *1*, Control lysosomes (not treated with cytosol); *2*, lysosomes treated with cytosol in the absence of GTP-γ-S; *3*, lysosomes treated with cytosol in the presence of 500 μM GTP-γ-S. (*B*) *1*, Control lysosomes; *2*, lysosomes treated as in line 3 of *A*. A typical result out of three measurements.

suggests that treatment of lysosomes with GTP-γ-S-activated cytosol increased permeability to H^+. The effect of GTP-γ-S-activated cytosol on lysosomal H^+ permeability was further examined using the protonophore CCCP. Since CCCP can make lysosomal membrane permeable to H^+ and produce an equilibrium membrane potential for H^+, an additional decrease in the fluorescence of $DiSC_3(5)$ can be produced by the agent during the measurement of lysosomal membrane potential (Harikumar & Reeves, 1983). As shown in Figure 4b, the degree of fluorescence quenching of cytosol-treated (in the presence of GTP-γ-S, line 2) lysosomes is larger than that of the control sample (line 1) in the absence of CCCP but the magnitude of CCCP-induced additional fluorescence quenching of the former is smaller than that of the latter. The results indicate that the proton permeability of the former approached the ion permeability produced by CCCP more closely than that of the latter. This reflects an increase in the proton permeability of cytosol-treated lysosomes.

Previous studies demonstrated that K^+/H^+ exchange plays an important role in the lysosomal uptake of K^+ and that the pH gradient across lysosomal membranes is the driving force for the exchange (Reeves, 1984). In order to reexamine whether external K^+ entered the cytosol-treated lysosomes through K^+/H^+ exchange, we lowered the lysosomal ΔpH and examined changes in the lysosomal uptake of K^+ by measuring the enzyme free activity increase.

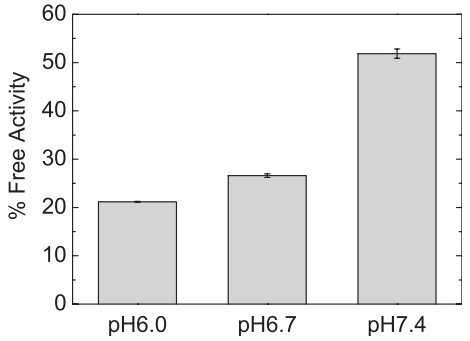

Fig. 5. Effects of K_2SO_4 medium pH on the integrity of cytosol-treated lysosomes. A 7.5-μl lysosomal sample was treated with 30 μl cytosol in the presence of 500 μM GTP-γ-S at 37°C for 10 min. Then, the sample was incubated in 120 μl 0.125 M K_2SO_4 medium (buffered at the indicated pH with 10 mM HEPES/citric acid or HEPES/KOH) at 37°C for 20 min. Lysosomal free enzyme activity was measured immediately after incubation. Values are means ± standard deviation of three measurements.

As shown in Figure 5, free enzyme activity of the cytosol-treated lysosomes (in the presence of GTP-γ-S) was 22%, 27% and 53%, respectively, after 20-min incubation in K_2SO_4 medium (buffered at pH 6.0, 6.7 and 7.4, respectively). Increasing the lysosomal ΔpH caused enzyme free activity increases in the K^+-containing medium. This suggests that K^+ entered the lysosomes via K^+/H^+ exchange.

Cytosolic PLC Increased Lysosomal Osmotic Sensitivity

Lysosomes are the intracellular "osmometer" because the organelle is sensitive to osmotic shock (Lloyd & Forster, 1986). Increases in their osmotic sensitivity can cause disruptions in lysosomal osmotic stress. As demonstrated above, the cytosol treatment increased the permeability of lysosomes to K^+ and H^+. Influx of K^+ can cause an osmotic imbalance across lysosomal membranes. Whether the lysosomes can stand such an osmotic stress depends to a large extent on their osmotic sensitivity. To examine whether the cytosol treatment increased lysosomal osmotic sensitivity, lysosomal integrity was examined after incubation in hypotonic sucrose medium. Treatment of lysosomes with GTP-γ-S-activated cytosol increased free enzyme activity markedly in hypotonic sucrose medium (Fig. 6, compare line 1 with lines 2 and 3). This suggests that the lysosomes lost their normal ability to resist hypotonic pressures; i.e., lysosomal osmotic sensitivity increased.

The enhancement of lysosomal osmotic sensitivity should induce an increased influx of water into the lysosomes during an osmotic stress, which can cause the lysosomes to become swollen. Therefore, changes in lysosomal size in an osmotic shock may provide direct evidence for increases in osmotic sensitivity. As

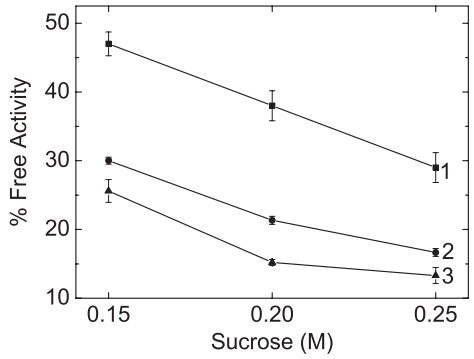

Fig. 6. Effects of sucrose concentration on the osmotic stability of lysosomes. A 7.5-μl lysosomal sample was treated with 30 μl cytosol in the presence of 500 μM GTP-γ-S or not at 37°C for 10 min. Then, the sample was incubated in sucrose medium at the indicated concentration at 37°C for 5 min. Lysosomal free enzyme activity was measured immediately after incubation. *1*, Lysosomes treated with cytosol in the presence of GTP-γ-S; *2*, lysosomes treated with cytosol in the absence of GTP-γ-S; *3*, control lysosomes (not treated with cytosol). Values are means ± standard deviation of three measurements.

visualized by the fluorescence of acridine orange inside lysosomes, the sizes of control (Fig. 7a) and cytosol (activated by GTP-γ-S)-treated lysosomes (Fig. 7c) are similar in isotonic sucrose medium. When examined in hypotonic sucrose medium, control (Fig. 7b) and cytosol-treated (without GTP-γ-S, Fig. 7e) lysosomes only slightly swelled during a 5-min treatment, while lysosomes treated by GTP-γ-S-activated cytosol enlarged markedly (Fig. 7d). The results suggest that the control lysosomes can resist an osmotic imbalance across their membranes within a short period. In other words, these lysosomes exhibited only limited osmotic sensitivity. The cytosol (activated by GTP-γ-S) treatment induced lysosomal enlargement, reflecting that the lysosomes lost their ability to stand the osmotic stress. It should be noted that although lysosomal osmotic sensitivity increased after the cytosol treatment, the lysosomes could still maintain their normal size under isotonic conditions (compare Fig. 7c with 7a). The results indicate that the lysosomes with higher osmotic sensitivity were not osmotically destabilized until an osmotic stress occurred. The influx of K^+ may produce osmotic stresses to the lysosomes and therefore destabilize them.

Discussion

Lysosomes normally maintain integrity in living cells. It is a prerequisite for the organelle to carry out its functions. However, lysosomes *in vivo* can be disintegrated under various conditions such as oxygen stress, necrosis, apoptosis and some diseases. In the past years, interest in the mechanism of lysosomal disintegration has heightened with the realization that this event is critical not only for organelle activities but also for cell viability. To clarify how lysosomes are disintegrated is important for the study of lysosome pathology, cell death and the pathogenesis of some diseases. Little information is available concerning the related mechanism. In this study, we established that GTP-γ-S-activated cytosol can disintegrate lysosomes by the actions of PLC.

Lysosomes can be disintegrated either by some injury-induced alterations in membrane structures or by osmotic stress-induced lysosomal destabilization. Lysosomal membrane lipid is an important component of the membrane barrier. Damage to the membrane phospholipids can disintegrate lysosomes (Zhang & Yao, 1997). A previous study demonstrated that hydrolyzing lysosomal membrane lipids by their internal phospholipase could destabilize isolated lysosomes (Weglicki et al., 1974). Increased uptake of external ions or molecules by lysosomes can cause osmotic imbalance across the membranes. The resultant osmotic stresses may disintegrate the lysosomes. Various lines of evidence indicate that the lipid portion of lysosomal membranes plays important roles in the regulation of their permeability. Changes in the physical states of lysosomal membrane lipids and photooxidation of the membrane lipids can increase lysosomal osmotic sensitivity and membrane permeability to K^+ (Yang et al., 2000). Lysosomes *in vivo* are surrounded by a high concentration of cytosolic K^+ (140 mM). A number of studies have emphasized that uptake of K^+ by lysosomes to some extent can cause osmotic shock and disrupt the organelle (Reeves, 1984). For this reason, we paid attention to the lysosomal permeability to K^+ when lysosomes were destabilized.

In normal cases, lysosomal membranes exhibit only limited permeability toward K^+ and H^+ (Harikumar & Reeves, 1983). This is favorable for lysosomal osmotic stability. The above results demonstrate that activated cytosol PLC increased lysosomal permeability to the ions, which osmotically destabilized the lysosomes via K^+/H^+ exchange. The increases in ion permeability might be attributed to the enzyme-produced destruction of membrane structures. As proposed previously, transient defects arising from thermal fluctuation in the membrane lipids is a major pathway for the movement of water and ions across membranes (Deamer & Bramhall, 1986). PLC can hydrolyze membrane phospholipids, resulting in the release of phosphate from phospholipids. It is likely that the PLC-produced defects at the polar head of lysosomal membrane phospholipids facilitate penetration of water into the membranes, which promotes K^+ diffusion across the membranes. Increases in the hydrogen bonds of water within membranes provide a pathway for the efflux of lysosomal H^+ (Marrink,

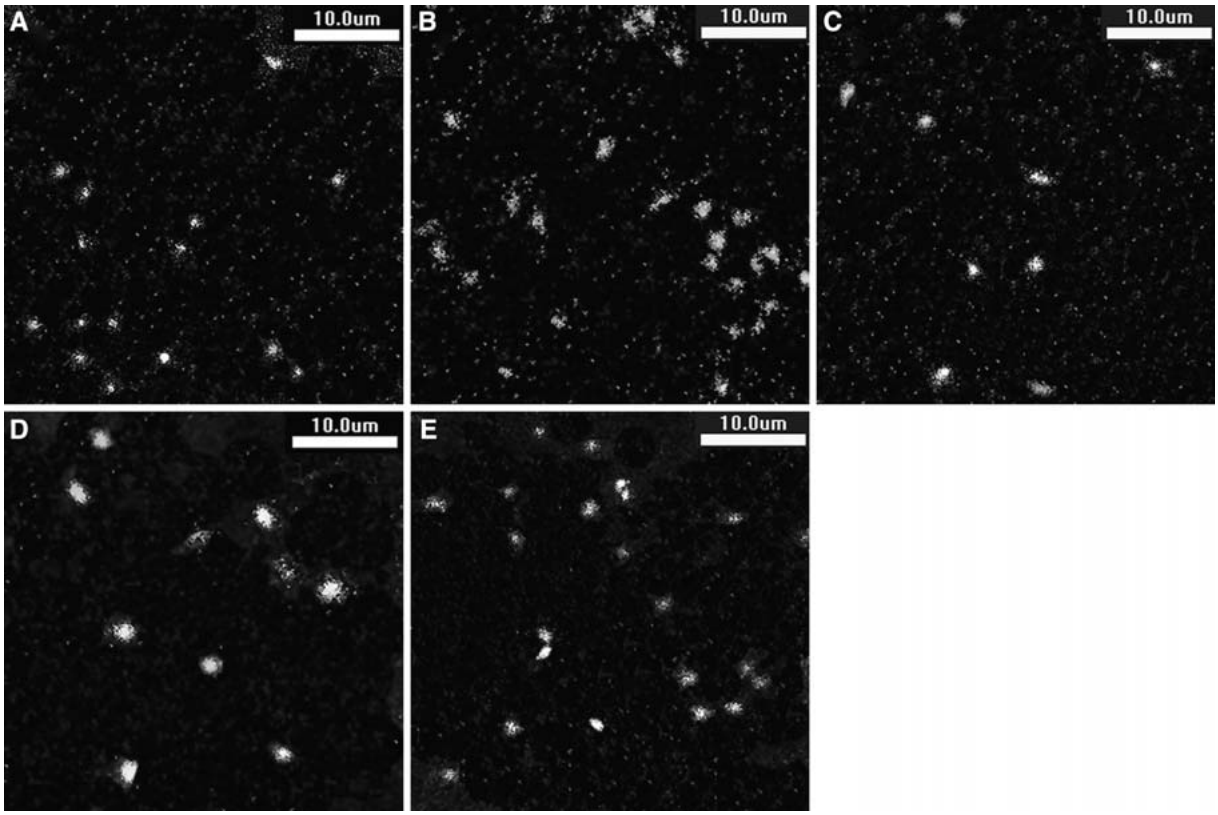

Fig. 7. Effects of cytosol and GTP-γ-S on lysosomal size. A 7.5-μl lysosomal sample was treated with 30 μl cytosol in the presence of 500 μM GTP-γ-S or not at 37°C for 5 min. Then, the sample was incubated in sucrose medium at the indicated concentrations at 37°C for 5 min. (*a*) Control lysosomes in 0.25 M sucrose medium. (*b*) Control lysosomes in 0.15 M sucrose medium. (*c*) Lysosomes treated with cytosol in the presence of 500 μM GTP-γ-S, then incubated in 0.25 M sucrose. (*d*) Lysosomes treated with cytosol in the presence of 500 μM GTP-γ-S, then incubated in 0.15 M sucrose. (*e*) Lysosomes treated with cytosol without GTP-γ-S, then incubated in 0.15 M sucrose. Detailed procedures were as described in Materials and Methods. A typical result out of three measurements is shown. Bars = 10 μm.

Jahnig & Berendsen, 1996). In addition to K^+-induced osmotic stress, the enzyme might cause defective membranes to become fragile. Thus, lysosomes are prone to lose their normal ability to resist osmotic stresses and to become disrupted.

Our recent study demonstrated that Ca^{2+}-activated cytosolic PLC could increase lysosomal osmotic sensitivity (Zhao et al., 2005). Although damaged lysosomal membranes are relatively permeable to water and become more fragile in osmotic stress, lysosomes are still stable under isotonic conditions. They lost integrity more markedly than normal lysosomes only in osmotic shock. The results of this study established that cytosolic PLC could produce an osmotic stress to the lysosomes via increased permeability to K^+.

Lysosomal destabilization is an abnormal event. Recently, we established that cytosolic PLC could increase lysosomal osmotic sensitivity after activating the enzyme by Ca^{2+} at about 340 nM. This suggests that a sustained elevation of cytosolic Ca^{2+} above normal concentrations (10–100 nM) may activate cytosolic PLC and destabilize lysosomes. A number of studies have 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 (Gasbarrini et al., 1992; Orrenius, Zhivotovsky & Nicotera, 2003). Whether lysosomes can be disintegrated by cytosolic phospholipases at normal cytosolic [Ca^{2+}] is unknown. As shown in this study, cytosolic PLC can destabilize lysosomes in the presence of GTP-γ-S at normal [Ca^{2+}]. The results suggest that cytosolic G protein played a role in the event, providing a new mechanism for lysosomal destabilization.

Small G proteins play important physiological rolls in various cellular activities, such as vesicle fusion and movement of the cytoskeleton. GTP-γ-S-induced release of lysosomal contents may play a role in lysosomal exocytosis (Chen et al., 2000). Previous studies demonstrated that small G proteins can activate phospholipases (Exton, 1994) and that GTP-γ-S can activate PLC in rabbit thymocytes (Behl et al., 1988). Recent studies show that small G proteins are activated and involved in the induction of apoptosis (Ueda et al., 2004; Su et al., 2005). In the early stage

of apoptosis, cathepsins leaked from destabilized lysosomes can induce apoptosis (Monney et al., 1998). However, how the lysosomes are disintegrated in apoptosis is not fully elucidated. Whether cytosolic G protein-activated PLC may disintegrate lysosomes in apoptosis requires further study.

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