Loss of membrane cholesterol influences lysosomal permeability to potassium ions and protons

Dong Deng [b], Nan Jiang [c], Shu-Jing Hao [a,d], Hui Sun [b], Guo-jiang Zhang [a,*]

[a] Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, PR China
[b] School of Life Sciences, Northeast Normal University, Changchun 130024, PR China
[c] Capital Normal University, Beijing 100037, PR China
[d] Graduate School, Chinese Academy of Sciences, Beijing 100039, PR China

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ABSTRACT

Cholesterol is an essential component of lysosomal membranes. In this study, we investigated the effects of membrane cholesterol on the permeability of rat liver lysosomes to K^+ and H^+, and the organelle stability. Through the measurements of lysosomal β-hexosaminidase free activity, membrane potential, membrane fluidity, intra-lysosomal pH, and lysosomal proton leakage, we established that methyl-β-cyclodextrin (MβCD)-produced loss of membrane cholesterol could increase the lysosomal permeability to both potassium ions and protons, and fluidize the lysosomal membranes. As a result, potassium ions entered the lysosomes through K^+/H^+ exchange, which produced osmotic imbalance across the membranes and osmotically destabilized the lysosomes. In addition, treatment of the lysosomes with MβCD caused leakage of the lysosomal protons and raised the intra-lysosomal pH. The results indicate that membrane cholesterol plays important roles in the maintenance of the lysosomal limited permeability to K^+ and H^+. Loss of this membrane sterol is critical for the organelle acidification and stability.

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1. Introduction

Lysosomes participate in the physiological turnover of cellular macromolecules such as proteins, lipids, carbohydrates and nucleic acids. In addition, the organelle plays important roles in a variety of cellular activities, including vesicle traffic, signal transduction, cell membrane repair and so on. To carry out these functions, lysosomes must maintain their integrity and provide favorable pH conditions for the various acidic hydrolases therein. The destabilization of lysosomes can collapse the pH gradient across their membranes and induce leakage of their hydrolases. These changes not only lead to lysosomal dysfunction but also bring about harmful effects in the pathogenesis of many diseases such as prion encephalopathies [1], Alzheimer's disease [2] and so on. Recent studies demonstrated that the leakage of lysosomal enzymes could also induce apoptosis and necrosis [3,4]. Therefore, identifying the membrane factors that affect lysosomal stability and elucidating the mechanism by which the lysosomes can be destabilized are important for the studies of cell pathophysiology [5,6].

Cholesterol is an essential component of cellular membranes. This sterol modulates various membrane properties such as membrane fluidity, permeability, stability and contour. In the lysosomal membrane, cholesterol is present in high concentration too [7]. However, the effects of cholesterol on the lysosomal membrane properties are not fully studied. As demonstrated previously, the lysosomal ion permeability is an important property of the organelle [8]. The lysosomal membrane permeability to protons greatly affects their internal acidification. In addition, the ion permeability also affects the influx of external cations by an electroneutral exchange. The lysosomes of mammalian cells are surrounded by a high concentration of cytoplasmic K^+ (140 mM) [9]. The increases in the lysosomal permeability to K^+ can osmotically disrupt the lysosomes through uptakes of K^+. In the past years, the lysosomal permeability to K^+ and H^+ was extensively studied. Most studies focused on the effects of external factors that affect the ion permeability, including temperature [8], oxidation [10], phospholipase [49], arachidonate [12] and so on. Cholesterol affects various properties of cytoplasma membrane. Whether the cholesterol of lysosomal membranes affects their permeability to K^+ and H^+ is still unknown. It is of interest to elucidate this issue. In this study, we established that the cholesterol of lysosomal membranes plays important roles in the maintenance of the organelle integrity. This sterol maintains the lysosomal permeability to K^+ and H^+ at low level. Loss of the cholesterol can increase

the lysosomal permeability to the ions, causing an influx of K$^+$ into the lysosomes via K$^+$/H$^+$ exchange. As a result, the lysosomes are destabilized.

2. Materials and methods

2.1. Materials

Carbonyl cyanide m-chlorophenylhydrazone (CCCP), 1,6-Diphenyl-1,3,5-hexatriene (DPH) fluorescein isothiocyanate-dextran (FITC-Dextran, $M = 70,000$), 4-methylumbelliferyl N-acetyl-beta-D-glucosaminide, methyl-β-cyclodextrin (MβCD) and valinomycin were from Sigma (St. Louis, MO). Bis (3-propyl-5-oxoisoxazol-4-yl) pentamethine-oxonol (oxonol VI) was from Molecular Probes. Percoll was purchased from Amersham (Uppsala, Sweden). Bicinchoninic acid kit (BCA) Protein Assay Kit was from PIERCE. Cholesterol assay kit was from BIOSINO BIO-TECHNOLOGY AND SCIENCE INC. Other chemicals were of analytical grade from Beijing Chemical Factory.

2.2. Preparation of lysosomes

Male Wistar rats were starved for 24 h and killed by decapitation. Rat liver lysosomes were isolated by the method of A.J. Jonas et al. [11]. 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 lysosome from mitochondria [13]. 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. The lysosomal fraction represented at least a 50-fold purification over the initial homogenate as determined by the specific activity of β-hexosaminidase. The recovery of lysosomes is 14.4%. The purified lysosomes were resuspended in 0.25 M sucrose medium at 1.35 mg membrane protein/ml for use. All performances were carried out at 4 °C.

2.3. Lysosomal membrane preparation

Control or treated lysosomes were centrifuged at 40,000 g for 10 min. The washed lysosomes were lysed for 30 min in a hypotonic solution containing 0.025 M sucrose/1 mM Tris–HCl, pH7.4 [14]. Then, the lysosomal membranes were collected by a centrifugation at 40,000 g for 30 min. All performances were carried out at 4 °C. Protein was quantified using bicinchoninic acid kit (Pierce) [15].

2.4. Treatment of lysosomes with methyl-β-cyclodextrin

The purified lysosomes were treated with MβCD at indicated concentration in 0.25 M sucrose or 0.125 M K$_2$SO$_4$ at 37 °C for the indicated time. The lysosomes in the treatment medium are at 52 μg membrane protein/ml.

2.5. Assay of lysosomal integrity

Lysosomal integrity was assessed by measuring the lysosomal enzyme latency. The latency of a lysosomal enzyme refers to the percent of intact lysosomes as revealed by the inability of substrate to reach the lysosomal enzyme until the organelles are deliberately ruptured [16]. 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide, the substrate of lysosomal β-hexosaminidase, was used at 1 mM to measure the enzyme activity [17]. The liberated 4-methylumbelliferone was determined by measuring its fluorescence (excitation 365 nm, emission 444 nm) on a Hitachi F-4010 fluorescence spectrophotometer. Activities of the enzyme measured in the absence and

presence of 0.36% Triton X-100 was designated the free activity and the total activity, respectively. Percentage free activity was calculated as (free activity / total activity) × 100%. Loss of lysosomal integrity was determined as increased percentage free activity.

2.6. Assay of lysosomal permeability to K$^+$

The lysosomal permeability to K$^+$ was assessed by the osmotic protection method. It is widely used to determine whether a solute can enter the lysosomes [8,18,19]. According to the principle of this method, an impermeable solute can provide perfect osmotic protection to the lysosomes suspended in the isotonic solution. However, the solute that penetrates into the lysosomes can break the initial osmotic balance across the lysosomal membranes. A progressive osmotic imbalance develops with increasing the solute concentration inside the lysosomes. As a result, the lysosomes swell and burst. Hence the permeability to a solute including ions can be monitored by measuring the changes in the lysosomal enzyme latency after incubating the lysosomes in the solution. This approach also gives a semiquantitative measure of relative rate of entry of permeant ions [20]. In short, 50 μl control or treated lysosomal sample was used for the assay of lysosomal enzyme latency. Enhancement of the lysosomal permeability to K$^+$ was determined as increased free lysosomal enzyme activity.

### 2.7. Measurement of lysosomal membrane potential

The permeability of lysosomes to K$^+$ can be assessed by measuring the lysosomal membrane potential using membrane potential-sensitive probe oxonol VI [21]. The assay medium contained 0.25 M sucrose (pH adjusted to 6.7 with imidazole), oxonol VI (2 μM) and CCCP (2 μM). 200 μl 0.75 M K$_2$SO$_4$ was added to the sample and the blank cuvettes at indicated time. Membrane potential was registered by the absorbance difference $\Delta A_{625-587}$ [22]. All measurements were performed on a Hitachi 557 spectrophotometer.

2.8. Measurement of lysosomal proton leakage

The leaked lysosomal protons can acidify the suspending medium. Based on the property of p-nitrophenol that the unprotonated molecules of the dye have a sufficiently larger extinction coefficient at 400 nm over that of protonated molecules, the acidification of assay medium induced by lysosomal proton leakage can be measured by monitoring the decrease in the p-nitrophenol absorbance at 400 nm [10,23]. The assay medium contained 0.25 M sucrose and 0.04 mM p-nitrophenol (Na salt). 80 μl lysosomal sample was added to 2 ml assay medium and then MβCD and CCCP were added to the medium. Absorbance of the pH sensitive dye p-nitrophenol was measured at 400 nm for 5 min on a UNIC-2100 spectrophotometer (Made in Shanghai, China).

2.9. Measurement of intra-lysosomal pH

According to the method of Ohkuma, the intra-lysosomal pH was measured using pH sensitive probe FITC-Dextran [24]. The fluorescence excitation spectrum of FITC-Dextran varies with pH. It is widely used to measure cell pH and organelle pH. Lysosomal pH can be measured directly by allowing cells to take up fluorescein isothiocyanate linked to high-molecular weight dextran. The advantages of this measurement of intravesicular pH are relatively rapid, not toxic, and subject to few artifacts [25]. Briefly, rat was injected intraperitoneally with FITC-Dextran (20 mg FITC-Dextran/150 g body weight) and starved for 16 h before decapitation. Lysosomes were prepared as described above. The measurement medium was composed of 0.25 M sucrose or 0.125 M K$_2$SO$_4$, buffered at pH 7.0 with 20 mM Hepes/Tris. Fluorescence was measured with excitation and emission
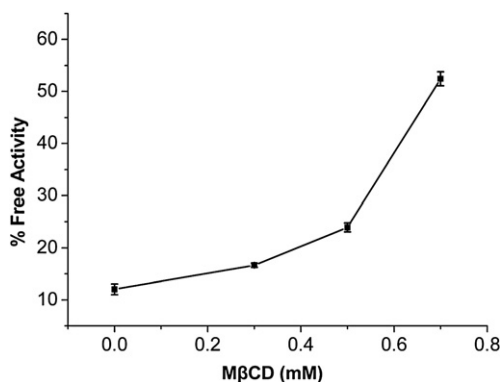
Fig. 1. MβCD treatment enhanced lysosomal permeability to potassium ions. Lysosomes were treated with indicated concentration of MβCD in 0.125 M K$_2$SO$_4$ (buffered at pH 7.0 with 20 mM Hepes/Tris) for 20 min. Then, lysosomal free enzyme activity was measured immediately. Increases in the free enzyme activity reflect increases in the lysosomal permeability to potassium ions. Values are means±S.D. of three measurements.

wavelengths of 495 and 550 nm, respectively, on a Hitachi F-4010 fluorescence spectrophotometer. Intra-lysosomal pH was calculated from the fluorescence intensity of the lysosomal sample relative to that after addition of Triton X-100 to 0.2%, using a calibration curve generated as described by Ohkuma [24]. In short, a calibration curve is constructed by measuring the intensity of fluorescence emission of FITC-Dextran in phosphate-buffered saline at various pH values.

2.10. Steady-state fluorescence anisotropy measurement

DPH labeling solution (2 μM) was prepared by diluting the tetrahydrofuran-dissolved DPH stocking solution (2 mM) with 0.1 M PBS buffer (pH 7.4) containing 0.1 M sucrose and stirring vigorously. For labeling, lysosomes were incubated in the labeling solution at 37 °C for 60 min. Fluorescence was measured on a Hitachi 4010 fluorescence spectrophotometer with excitation and emission at 350 and 452 nm, respectively. Steady-state fluorescence anisotropy (r) was calculated according to the equation [26]:

$$\gamma = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$$

where I_{VV} and I_{VH} are the fluorescence intensities measured with the excitation polarizer in the vertical position and the analyzing emission polarizer mounted vertically and horizontally, respectively. $G = I_{HV}/I_{HH}$ is the correction factor. Correction for light scattering was carried out as described by Litman [27]. As pointed by Blitterswijk [28], high degrees of fluorescence anisotropy indicate higher degrees of membrane order or lower degrees of membrane fluidity, and vice-versa.

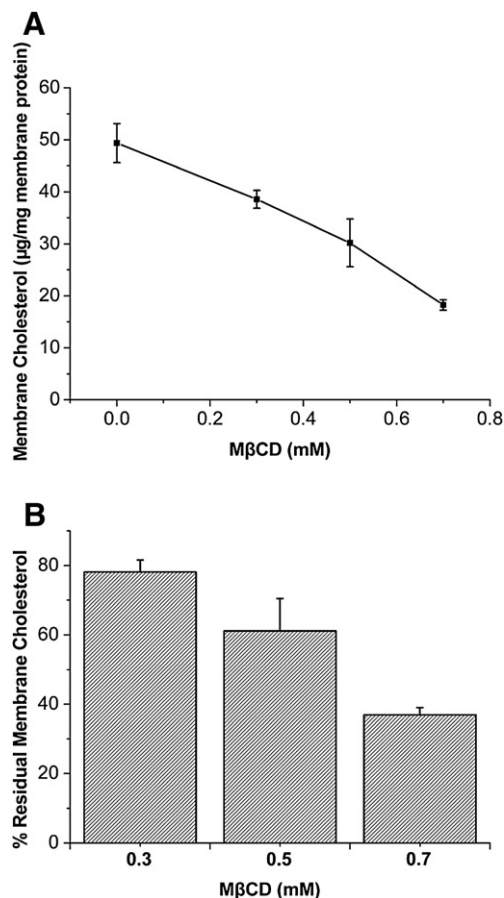### 2.11. Assay of lysosomal membrane cholesterol

Lysosomal membrane was prepared as described above. According to the method of Heider [29], lysosomal membrane cholesterol was extracted with isopropyl alcohol. In short, isopropyl alcohol was mixed with lysosomal membranes at 0.3 ml/mg membrane protein and sonicated for 10 min. Then, the sample was centrifuged at 20,000 g for 5 min. The supernatant was used for cholesterol analysis. Cholesterol content was quantified using cholesterol assay kit (BIOSINOBIO-TECHNOLOGY AND SCIENCE INC).

3. Results

The limited permeability of lysosomes to K$^+$ is favorable for the maintenance of their osmotic stability [30]. In order to study the effect of membrane cholesterol on the lysosomal permeability to K$^+$, we treated the lysosomes with MβCD to deplete their membrane

cholesterol [26] and assessed the changes in the membrane permeability to K$^+$ using osmotic protection method [20,31]. In this assay, the lysosomes were suspended in an isotonic K$^+$-containing medium (0.125 M K$_2$SO$_4$). The entry of K$^+$ into the lysosomes will cause an osmotic imbalance across their membranes. The increase in the lysosomal K$^+$ permeability produces progressive accumulation of K$^+$ inside the lysosomes, which causes the lysosomes to swell and a loss of their integrity. Thus, the lysosomal permeability to K$^+$ can be assessed by measuring the lysosomal enzyme latency [8,18,19]. As shown in Fig. 1, the free enzyme activity of the lysosomes increased from 12% to 52% with increasing MβCD concentration to 0.7 mM during 20-min incubation in the K$_2$SO$_4$ medium. In contrast to the value obtained in the K$_2$SO$_4$ medium, free enzyme activity of 0.7 mM MβCD-treated lysosomes was only 15% after 20-min incubation in 0.25 M sucrose (data not shown). It implies that the MβCD treatment itself did not destabilized the lysosomes. According to the principle of osmotic protection method, the loss of lysosomal latency in the K$_2$SO$_4$ medium reflects an influx of K$^+$. It indicates that treating the lysosomes with MβCD increased their permeability to K$^+$. Since MβCD can selectively deplete membrane cholesterol, the dependence of lysosomal latency loss on the MβCD concentration suggests that the increases in the lysosomal K$^+$ permeability was probably due to the loss of membrane cholesterol.

Additional experiments were carried out to examine whether the MβCD treatment decreased the lysosomal membrane cholesterol. As shown in Fig. 2A, the lysosomal membrane cholesterol decreased from 49.4 μg/mg membrane protein to 18.2 μg/mg membrane protein with
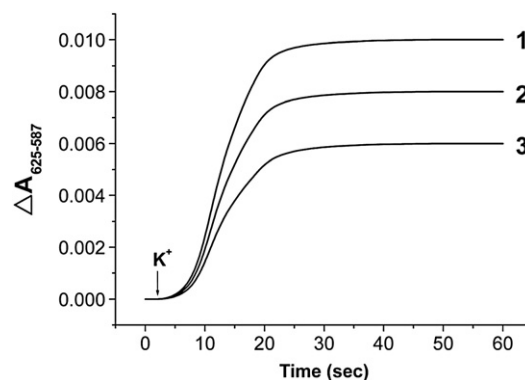

Fig. 2. Effect of MβCD treatment on lysosomal membrane cholesterol content. Lysosomes were treated with MβCD for 20 min. Then, lysosomal membrane cholesterol was measured. (A) Lysosomal membrane residual cholesterol; (B) residual lysosomal membrane cholesterol was expressed as a percentage of the control values. Values are means±S.D. of three measurements.

increasing MβCD concentration in the treatment. The membrane cholesterol decreased by 21.9%, 39.9% and 63.1% after the lysosomes were treated with 0.3, 0.5 and 0.7 mM MβCD, respectively (Fig. 2B). The results suggest that loss of the membrane cholesterol may increase the lysosomal membrane permeability to K^+.

Cholesterol is an important modulator of membrane physical state [32]. Since the membrane fluidity of lysosomes can affect their permeability to K^+ [33], we examined the effect of MβCD on the lysosomal membrane fluidity. The results show that treatment of the lysosomes with MβCD significantly reduced the fluorescence anisotropy of DPH (Table 1). It indicates that the lysosomal membrane fluidity increased. Our previous results showed that the fluidization of lysosomal membranes could decrease their permeability to K^+ [33]. These results suggest that the MβCD-induced increase in the lysosomal K^+ permeability was caused by the loss of the membrane cholesterol but not by the increase in the membrane fluidity. In addition, increasing the MβCD concentration could increase the ion permeability further (cf. Fig. 1) but did not fluidize the membrane further (cf. Table 1, anisotropy of the samples treated by 0.3, 0.5 and 0.7 mM MβCD was similar). It suggests that the effect of MβCD on the lysosomal K^+ permeability was not correlated to the fluidization of the lysosomal membranes.

The effect of MβCD on the lysosomal K^+ permeability was further assessed by the measurement of membrane potential using oxonol VI as a probe [21,34]. This membrane potential-sensitive dye has been used to indicate the relative permeability of K^+ through its absorbance changes. An increase in the differential absorbance ($\Delta A_{625-587}$) of oxonol VI will be observed when potassium ions are allowed to enter the lysosomes [21]. As shown in Fig. 3, the differential absorbance of the MβCD-treated lysosomes (line 2) increased more markedly than that of the control lysosomes (line 3) upon addition of K^+ to the medium. It implicates that the MβCD treatment enhanced the lysosomal K^+ permeability. Compared with the control lysosomes (line 3), the dye absorbance of the MβCD-treated lysosomes (line 2) is closer to the valinomycin-permeabilized lysosomes (line 1). It indicates that the ion permeability of the MβCD-treated lysosomes approximated to that of the valinomycin-permeabilized lysosomes. This result further established that the MβCD treatment increased the lysosomal permeability to K^+. It has been established that lysosomal protons produce an inside negative membrane potential [30]. Because the fluidization of lysosomal membranes may increase their proton permeability and therefore increase the membrane potential [23], we used the protonophore CCCP to permeabilize the lysosomes to protons. Thus, the measured membrane potential depended solely on the changes in the membrane permeability to K^+.
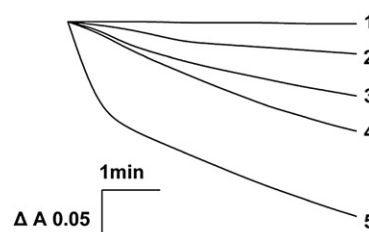
Normal lysosomes exhibit a limited permeability toward H^+, which is favorable for the maintenance of intralysosomal acidic pH [30]. The increases in the lysosomal H^+ permeability can cause leakage of their protons, which may acidify the lysosomal suspending medium and can be assessed with pH-sensitive dye p-nitrophenol [23]. Using this method, we studied the effect of MβCD on the lysosomal proton permeability. As shown in Fig. 4, the absorbance of the MβCD-treated lysosomal samples decreased more markedly (lines 3 and 4) than that

Fig. 3. Effects of MβCD treatment on the lysosomal membrane potential. Lysosomes were treated with 0.5 mM MβCD for 5 min. Then, membrane potential of MβCD-treated or control lysosomes was measured immediately. Assay medium contained 0.25 M sucrose (pH adjusted to 6.7 with imidazole), 2 μM oxonol VI and 2 μM CCCP. 200 μl 0.75 M K_2SO_4 was added at the indicated time. Membrane potential was registered by the absorbance difference $\Delta A_{625-587}$. Line 1: control lysosomes, assay medium contained 1 μM valinomycin; line 2: MβCD-treated lysosomes; line 3: control lysosomes, not treated with MβCD. A typical result from three experiments is shown.

of the control lysosomes (line 2). Because the unprotonated p-nitrophenol molecules have a sufficiently larger extinction coefficient at 400 nM over that of the protonated molecules [23], the larger absorbance decrease of the MβCD-treated lysosomes reflected a greater proton leakage. The abolishment of the absorbance decrease in the buffered medium (compare lines 3 and 4 with line 1) confirmed the effect of proton leakage on the dye absorbance. Using CCCP to permeabilize lysosomes to H^+, we recorded a more marked absorbance decrease (line 5). It further established that the lysosomal proton leakage decreased the dye absorbance. These results indicate that the MβCD treatment increased the lysosomal H^+ permeability.

Leakage of lysosomal protons can elevate the intra-lysosomal pH. The effect of MβCD on the lysosomal H^+ permeability was further studied through the measurement of the lysosomal internal pH. As shown in Table 2, the control lysosomes maintained their pH in the sucrose medium. The intra-lysosomal pH increased only by 0.15 U in the K_2SO_4 medium, showing that the lysosomal protons leaked out at a low level and the exchange of the lysosomal H^+ for the external K^+ was limited. It is consistent with previous conclusion that normal lysosomes exhibit a limited permeability toward H^+ and K^+ [30]. In contrast to the values obtained in the sucrose medium, the intra-lysosomal pH increased by 0.27 U (for 0.5 mM MβCD treatment) and 0.62 U (for 0.7 mM MβCD treatment) in the K_2SO_4 medium. It suggests that the MβCD treatments caused marked leakages of the lysosomal protons in the K^+-containing medium. The enhancement of the lysosomal proton leakage is presumably due to an increased K^+/H^+

Table 1
Effect of MβCD treatment on lysosomal membrane fluidity

Treatment of lysosomes	Anisotropy (γ)	P
Control	0.204±0.006	–
0.3 mM MβCD	0.184±0.004	<0.05
0.5 mM MβCD	0.182±0.004	<0.001
0.7 mM MβCD	0.178±0.003	<0.001

Lysosomes were treated with MβCD for 20 min. Then, control and MβCD-treated lysosomes were labeled with 2 μM DPH. Lysosomal membrane fluidity was assessed by measuring DPH fluorescence anisotropy (γ). All procedures were as described in "Materials and methods". Values are means±S.D., $n=4$. Statistical analysis was performed using Student's t-test.

Fig. 4. MβCD treatment caused lysosomal proton leakage. All assay media of lysosomal proton leakage contained 0.25 M sucrose (adjusted to pH 7.0 with KOH) and 0.04 mM p-nitrophenol. In addition, the assay medium of line 1 contained 0.5 mM MβCD and buffered at pH 7.0 with 20 mM Hepes/Tris. Assay media of lines 3 and 4 contained 0.5 and 0.7 mM MβCD, respectively. No MβCD was added to the assay media of lines 2 and 5. The assay medium of line 5 contained 1 μM CCCP. Absorbance of p-nitrophenol was measured at 400 nm upon addition of 80 μl lysosomal sample to 2 ml assay medium. A typical result from three measurements is shown.

Table 2
Effects of MβCD treatment on the intra-lysosomal pH

Treatment of lysosomes	Treatment medium	pH (0 min)	pH (5 min)
Control	Sucrose	5.45±0.01	5.48±0.01
0.5 mM MβCD	Sucrose	5.45±0.01	5.54±0.01
0.7 mM MβCD	Sucrose	5.45±0.01	5.62±0.03
Control	K_2SO_4	5.46±0.01	5.61±0.01
0.5 mM MβCD	K_2SO_4	5.46±0.01	5.73±0.04
0.7 mM MβCD	K_2SO_4	5.45±0.01	6.07±0.05

Lysosomes were treated with MβCD in 0.25 M sucrose or 0.125 M K_2SO_4 (both buffered at pH 7.0 with 20 mM Hepes/Tris) for the indicated time. Then, the intra-lysosomal pH was measured as described in "Materials and methods". Values are means±S.D. of three measurements.

Table 3
Effect of MβCD treatment on lysosomal membrane fluidity

Treatment of lysosomes	Anisotropy (γ)	P
Control	0.202±0.008	–
0.5 mM MβCD	0.184±0.002	<0.05
0.7 mM MβCD	0.182±0.002	<0.05

Lysosomes were treated with MβCD for 5 min. Then, control and MβCD-treated lysosomes were labeled with 2 μM DPH. Lysosomal membrane fluidity was assessed by measuring DPH fluorescence anisotropy (γ). All procedures were as described in "Materials and methods". Values are means±S.D., $n=4$. Statistical analysis was performed using Student's t-test.

exchange. The effect of K^+/H^+ exchange on the lysosomal pH was confirmed by the result that the internal pH of the MβCD-treated lysosomes increased only by 0.09 U (for 0.5 mM MβCD treatment) and 0.17 U (for 0.7 mM MβCD treatment) in the sucrose medium. The values are much smaller than that measured in the K_2SO_4 medium (0.27 and 0.62 U). The slight increase of the lysosomal pH in the sucrose medium is possibly caused by an electroneutral co-efflux of the lysosomal H^+ with the intra-lysosomal anions such as chloride and phosphate [35]. These results support the above conclusion that the MβCD treatment increased the lysosomal H^+ permeability.

To study the effects of membrane cholesterol and membrane fluidity on the lysosomal H^+ permeability, we measured the lysosomal membrane cholesterol and fluidity under the same MβCD treatment conditions. As shown in Fig. 5A, the lysosomal membrane cholesterol
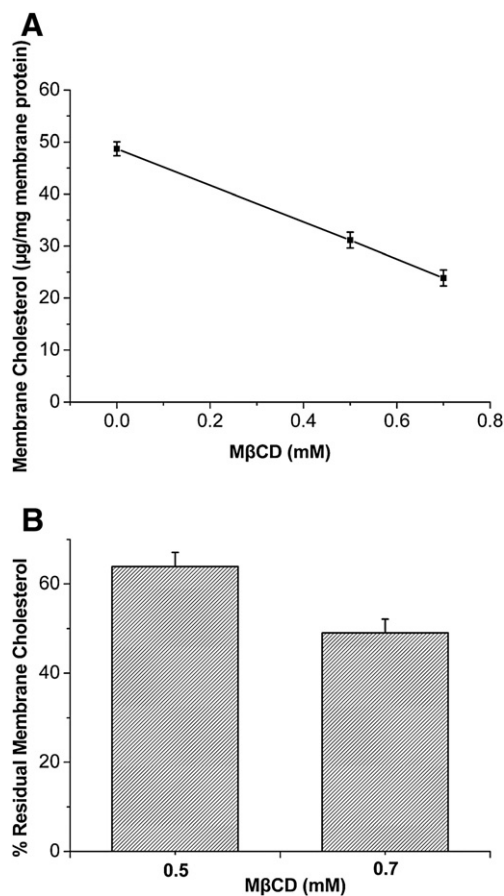
Fig. 5. Effect of MβCD treatment on lysosomal membrane cholesterol content. Lysosomes were treated with MβCD for 5 min. Then, lysosomal membrane cholesterol was measured. (A) Residual lysosomal membrane cholesterol; (B) residual lysosomal membrane cholesterol expressed as a percentage of control lysosomes. Values are means±S.D. of three measurements.

decreased from 48.7 μg/mg membrane protein to 23.9 μg/mg membrane protein after a treatment with 0.7 mM MβCD. The membrane cholesterol decreased by 36.1% and 51% after treating the lysosomes with 0.5 and 0.7 mM MβCD, respectively (Fig. 5B). Treatment of the lysosomes with 0.5 and 0.7 mM MβCD also reduced the fluorescence anisotropy of DPH markedly (Table 3), reflecting that the lysosomal membranes were fluidized. Since the fluorescence anisotropy values of 0.5 and 0.7 mM MβCD-treated lysosomes are similar, the lysosomal membranes were not fluidized further when MβCD was increased from 0.5 mM to 0.7 mM. However, increasing MβCD from 0.5 mM to 0.7 mM could increase the lysosomal proton leakage and internal pH further (Fig. 4 and Table 2). It suggests that the loss of membrane cholesterol but not membrane fluidization played a major role in the increase in the lysosomal H^+ permeability.

According to the electroneutral principle, the passive diffusion of K^+ into lysosomes must be accompanied by the charge-compensating ions to maintain the electroneutrality of the ion movement. As demonstrated previously, K^+/H^+ exchange is a major pathway for the passive diffusion of K^+ into lysosomes [20]. To what extent K^+ enters lysosomes correlates to the lysosomal permeability toward both K^+ and H^+. As shown above, the MβCD treatment caused the lysosomal protons to leak out through K^+/H^+ exchange. To determine whether potassium ions entered the MβCD-treated lysosomes via K^+/H^+ exchange, the lysosomes were suspended in the K^+-containing mediums that were buffered at different pH. The different pH gradient across the lysosomal membranes provided different driving force for the exchange of the lysosomal protons with the external K^+. Since the MβCD treatment increased the lysosomal permeability to H^+ and K^+, a larger ΔpH should cause a greater influx of K^+ into the lysosomes. The resulted osmotic imbalance should destabilize the lysosomes. As show in Fig. 6, the free enzyme activity of the MβCD-treated lysosomes increased markedly with elevating the medium pH from 6.6 to 7.4. It reflects that larger amounts of K^+ entered the lysosomes at higher pH of the medium. In contrast, the control lysosomes maintained their
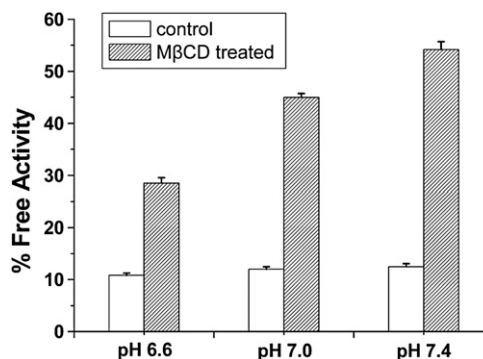
Fig. 6. Effects of medium pH on the integrity of MβCD-treated lysosomes. Lysosomes were treated with 0.7 mM MβCD in 0.125 M K_2SO_4 for 20 min. The K_2SO_4 medium was buffered at indicated pH with 20 mM Hepes/Tris. Lysosomal free enzyme activity was measured immediately after the treatment. Open bar: control lysosomes, not treated with MβCD; hatched bar: lysosomes were treated with MβCD. Values are means±S.D. of three measurements.

free enzyme activity at higher pH of the medium. The results suggest that K^{+} entered the MβCD-treated lysosomes through K^{+}/H^{+} exchange pathway.

4. Discussion

The lysosomal permeability to K^{+} and H^{+} is an important property of the organelle. The changes in the property may cause losses of the lysosomal functions and even lysosomal destabilization. The lysosomes *in vivo* are surrounded by 140 mM cytoplasmic K^{+}. To what extent the potassium ions enter lysosomes correlates to their permeability to K^{+}. Normal lysosomes show only a limited permeability toward K^{+} [30]. However, some factors or events such as low temperature [8], oxidation to lysosomal membranes [10], phospholipase [49] and some products of their catalyzed phospholipid hydrolysis [12,36,37] can increase the lysosomal permeability to K^{+}. The rigidification of lysosomal membranes can also increase the ion permeability [33]. As demonstrated previously, the increases in the influx of K^{+} into the lysosomes could osmotically disintegrate the organelle [31]. In the past, the influences of various factors on the lysosomal K^{+} permeability were extensively studied, but little is known about the effects of membrane cholesterol on the ion permeability. To elucidate this issue is important for the studies of cellular and lysosomal pathophysiology.

The H^{+}-ATPase-mediated proton translocation acidifies the lysosomal interior, which provides favorable pH for the acidic hydrolases therein. Normal lysosomes show a limited permeability toward H^{+} [30]. This property plays an important role in the maintenance of lysosomal acidification. The enhancement of lysosomal H^{+} permeability can directly affect the intra-lysosomal acidic pH and indirectly affect the lysosomal integrity. Previous studies indicated that the K^{+}/H^{+} exchange is a major pathway for the passive diffusion of K^{+} into the lysosomes [20]. According to the electroneutral principle, the passive diffusion of K^{+} into lysosomes must be accompanied by the charge-compensating ions to maintain the electroneutrality of the ion movement. It means that the entry of K^{+} into lysosomes must be accompanied by an entry of negative charge such as Cl^{−} or an efflux of positive charge such as H^{+}. By now, no evidence is available concerning the protein responsible for the K^{+}/H^{+} exchange in the lysosomes. The above mentioned K^{+}/H^{+} exchange refers to an electroneutral exchange of the diffusion of external K^{+} with internal H^{+}. Obviously, an increase in the lysosomal H^{+} permeability may promote the passive diffusion of K^{+} into lysosomes through the K^{+}/H^{+} exchange. Our recent studies demonstrated that phospholipase A₂ [38], phospholipase C [49], lysophospholipid [36], phosphatidic acid [37] and arachidonate [12] can increase the lysosomal permeability to H^{+}. In addition, we have established that the physical state of membranes [23] and the thiol groups of membrane proteins [39] also affect the ion permeability. However, the relations between the lysosomal membrane components and the lysosomal ion permeability are still unclear. In this work, we established that the loss of membrane cholesterol could increase the lysosomal permeability to K^{+} and H^{+}. The increase in the uptake of K^{+} could destabilize the lysosomes.

Cholesterol is an essential component of membranes. This class of lipid regulates various membrane properties such as membrane fluidity, permeability, stability and contour [40,41]. It also modulates membrane receptor function [26]. Cholesterol can interact with the phospholipids of membranes, forming specially separated domain named raft. Such raft plays important roles in many cellular processes including signal transduction, membrane trafficking, cytoskeletal organization, and pathogen entry [42]. Lysosome is one of the cholesterol pools in living cells. As demonstrated previously, the lysosomal membranes contain abundant cholesterol. Several cholesterol-binding proteins such as SCP-2 (Sterol Carrier Protein-2) can increase lysosomal membranes cholesterol exchange and the cholesterol of lysosomal membranes was rapidly altered [7]. Moreover, what role does cholesterol play in the lysosomal membranes is not well studied. Recently, a study demonstrated that the treatment of lysosomes with MβCD caused changes in the dynamic distribution of LAMP-2A, which enhanced the chaperone-mediated autophagy (CMA) activity [43]. In this study, we established that loss of the lysosomal membranes cholesterol could increase the permeability to K^{+} and H^{+}, which caused an influx of K^{+} into the lysosomes.

As proposed previously, cholesterol can reduce K^{+} permeability [44]. How the cholesterol decreases K^{+} permeation across membrane is unclear. Solubility-diffusion theory has been proposed to account for solute permeation of membranes. To cross the membrane by this mechanism, the permeating particle must partition into the hydrophobic region of the membrane, diffuse across, and leave by redissolving into the second aqueous phase. In this process, Born energy is required to transfer water or other charged particle from the high dielectric aqueous phase to the low dielectric membrane interior because the electrostatic energy of the ion is much lower in a water medium with high dielectric constant (∼80) than in a typical bilayer with low dielectric constant (∼2) [45]. Since cholesterol causes a significant drop in the dielectric constant in the region of the ester carbonyl groups of phospholipids [46], a relative larger Born energy is required for the ions to enter membranes. It is likely that loss of membrane cholesterol may increase the dielectric constant of membrane interior and decrease the Born energy required for an ion to cross the membrane. Thus, it becomes easier for ions such as K^{+} to pass through a membrane when its cholesterol content is decreased.

Cholesterol can modulate membrane physical state. It decreases the membrane order in the gel phase and increases the membrane order in the liquid-crystalline phase [47]. The phase transition of lysosomal membrane occurs at about 15 °C [31]. Thus, loss of membrane cholesterol should increase the lysosomal membrane fluidity above 15 °C. In this study, we obtained similar results. Various lines of evidence indicate that the physical state of membranes affects their ion permeability. Our previous studies established that increasing lysosomal membrane fluidity decreased their permeability to K^{+} [33] and that the lysosomal K^{+} permeability increased with rigidifying their membrane [21]. However, the above results show that loss of membrane cholesterol increased the lysosomal K^{+} permeability as the membrane was fluidized. Thus, reducing membrane cholesterol overpowered the effect of membrane fluidization on the ion permeability. It suggests that loss of membrane cholesterol played a major role in the increase of the lysosomal K^{+} permeability.

By now, there is no evidence showing the existence of K^{+} channel, K^{+} carrier and K^{+} pump on the lysosomal membranes. Our recent works also failed to find the channel. As demonstrated by the studies of lysosomal K^{+} permeability, K^{+} entered lysosomes by a passive diffusion mechanism [20,31]. To examine whether K^{+} entered the MβCD-treated lysosomes through K^{+} channel, we used potassium channel blockers 4-Aminopyridine, Tetraethylammonium and Quinine in the MβCD treatment of lysosomes. The results show that all of these blockers could not abrogate the effect of MβCD on the lysosomal K^{+} permeability (data not shown). It suggests that K^{+} entered the MβCD-treated lysosomes by a passive diffusion mechanism.

As proposed by Deamer and Nichols [48], a small fraction of water in membranes might be aggregated through hydrogen bonding, which provides a conductance pathway unique to protons. It means that protons penetrate membranes along the hydrogen bonds of water. Cholesterol can reduce the passive water permeability in direct proportion to its level in the membrane and therefore reduce proton permeation across membranes [44]. It explains why the loss of membrane cholesterol could increase the lysosomal permeability to H^{+}. Our previous study established that the fluidization of lysosomal membranes could increase their permeability to H^{+}. It suggests that the lysosomal membrane fluidization induced by the loss of

cholesterol might also increase the lysosomal permeability to H^+. Finally, membrane cholesterol can reduce the incidence of defects in membranes and therefore decrease ion permeability [41]. It is likely that loss of membrane cholesterol may increase ion permeability through increased membrane defects.

Acknowledgements

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