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Influence of Membrane Physical State on the Lysosomal Proton Permeability

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Abstract. Influence of membrane physical state on the proton permeability of isolated lysosomes was assessed by measuring the membrane potential with 3,3′-dipropylthiadicarbocyanine iodide and monitoring their proton leakage with *p*-nitrophenol. Changes in the membrane order were examined by the steady-state fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene. Both the membrane potential and proton leakage increased with fluidizing the lysosomal membranes by benzyl alcohol and decreased with rigidifying the membranes by cholesteryl hemisuccinate. The proton permeability increased to the maximum of 42% by the benzyl alcohol treatment and decreased to the minimum of 38.1% by the cholesteryl hemisuccinate treatment. Treating the lysosomes with protonophore CCCP increased the proton permeability by 58%. The effects of the membrane fluidization and rigidification can be reversed by rigidifying the fluidized membranes and fluidizing the rigidified membranes, respectively. The results indicate that the proton permeability of lysosomes increased and decreased with increasing and decreasing their membrane fluidity, respectively. Moreover, the lysosomal proton permeability did not alter further if the changes, either an increase or a decrease, in the fluidity exceeded some amount. The results suggest that the proton permeability of lysosomes can be modulated finitely by the alterations in their membrane physical state.

Key words: Lysosomes — Membrane fluidity — Proton permeability — Membrane potential — Proton leakage

Introduction

Lysosomes participate in the physiological turnover of cellular macromolecules such as nucleic acids, proteins, lipids and carbohydrates. In the past years, interest in the mechanism of lysosomal acidification has heightened with the realization that the acidic interior of lysosomes is favorable for the activities of their various acidic hydrolases, that these acidic hydrolases are responsible for the digestions of the macromolecules therein [36, 41], and that the pH gradient of lysosomes plays an important role in some carrier-mediated transports across their membranes [40]. By now, it is widely accepted that the maintenance of intralysosomal pH in vivo may be due to the dynamic equilibrium between the influx of protons by H^+-ATPase, their efflux in exchange for the monovalent cations in the cytoplasm and a Donnan equilibrium resulting from the intralysosomal impermeable negative charges contributed by acidic glycoproteins (perhaps also by acidic phospholipids and sialic acids) which must be balanced by an equal number of positive counterions including protons [34, 36]. Whether the lysosomes can be appropriately acidified and the acidic intralysosomal pH can be maintained is of the utmost importance for the pathophysiological properties of the organelle. Since the lysosomes in vivo are surrounded by a high concentration of cytoplasmic K^+ (140 mM) [1], the oppositely directed transmembrane concentration gradients of H^+ and K^+ can drive an exchange of lysosomal H^+ for the external K^+, therefore resulting in an elevation of the intralysosomal pH. Moreover, the H^+/K^+ exchange may osmotically destabilize the lysosomes by the entry of K^+ [5, 41, 57, 59]. In addition to the effects on the acidification and osmotic stability of lysosomes, the outward diffusion of lysosomal protons, which must be accompanied by charge-compensating migrations of either cations or anions, may play a role in the lysosomal ion homeostasis. Although the proton permeability of lysosomes affects their various activities, little is known about the membrane natures relating to such a property. The lysosomal membrane is relatively impermeable to H^+ in normal case compared with its permeability to some monovalent cations such as Cs^+, K^+

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and Na^+ [5, 41] but exhibits an increased permeability toward H^+ after the lysosomes are photodamaged [57] or treated by the antineoplastic drug lonidamine [9]. A number of investigators described some general features of the lysosomal proton permeability with respect to the order of their permeability to some monovalent cations such as H^+, Cs^+, Rb^+, K^+, Na^+ and Li^+ [5, 17]. By now, it is not clear what factor and/or component of lysosomal membranes modulate their permeability to H^+ and how the photodamage and lonidamine treatment increase the efflux of lysosomal protons. Further studies are needed to elucidate these issues.

There is considerable evidence that a series of membrane activities are influenced by the physical state of the membranes [47]. On the other hand, the membrane lipid fluidity can be modulated by a wide range of physiological variables [2, 10, 19, 20, 22, 33, 38, 39, 46]. As demonstrated previously, the proton permeability of some membranes is affected by the changes in their fluidity [3, 42, 43]. Since proton permeability is an important property for the lysosomes and the fluidity of their membranes is liable to change under some physiological and pathological conditions [21, 35, 48, 59], it may have pathophysiological significance to clarify if lysosomal membrane fluidity affects their proton permeability. In a number of studies, the effects of the bilayer lipid fluidity on the membrane activities and properties were studied after the membranes were treated with fluidity-modulating agents such as benzyl alcohol (BA) and cholesteryl hemisuccinate (CHS). Benzyl alcohol has been used as a membrane fluidizer [3, 15]. The agent is a neutral compound, and this precludes any selective interaction with charged lipid species and as such is a suitable tool to study the relationship between bilayer fluidity and membrane-associated functions [15]. Membrane rigidification can be achieved by incorporation of hydrophilic cholesterol ester CHS [58]. In the present work, we modulated the lysosomal membrane fluidity using these agents and measured the membrane lipid order which reflects the membrane fluidity by the widely used method of fluorescence anisotropy measurement [13, 50]. The results show that the proton permeability of lysosomes can be increased and decreased by increasing and decreasing their membrane fluidity, respectively. The mechanism for such effects is discussed.

Materials and Methods

Chemicals

$DiSC_3(5)$ was purchased from Molecular Probes (Eugene, OR); CCCP, Valinomycin, DPH, CHS and Hepes were from Sigma (St. Louis, MO). The other chemicals used were of analytical grade from local sources. All aqueous solutions were prepared with deionized, glass-distilled water.

Preparation of Lysosomes

Male wistar rats starved for 24 h were killed by decapitation. Rat liver lysosomes were prepared by the method of Ohkuma et al. [37]. All procedures were carried out at 0–4°C. Lysosomes were resuspended in 0.25 M sucrose medium at a final protein concentration of 12.5 mg/ml. Protein was determined according to Lowry et al. [26].

Modulation of Lysosomal Membrane Fluidity

Lysosomal membrane fluidity was modulated by the treatments with membrane fluidizer BA and rigidifier CHS [3, 15, 58]. CHS stock solution was prepared by the method of Wilbrandt et al. [58]. For fluidizing the membranes, isolated lysosomes were incubated in the presence of 20 mM BA at 37°C for indicated time. To decrease the fluidity of BA-treated lysosomal membranes, the above incubation was continued for indicated time upon addition of 0.025 mM (final concentration) CHS to the suspension. Rigidification of the membranes was accomplished by incubating the isolated lysosomes in the presence of 0.025 mM CHS at 37°C for indicated time. To increase the fluidity of CHS-treated lysosomal membranes, the above incubation was continued for indicated time upon addition of 20 mM (final concentration) BA to the incubation medium. Control samples were prepared by incubating the isolated lysosomes in the absence of BA or CHS, but BA or CHS was added to the samples at a final concentration same as that of the BA or CHS treated sample just before DPH labeling or the measurements of membrane potential and proton leakage.

Steady-State Fluorescence Anisotropy Measurement

DPH labeling solution (4 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, lysosomal samples were incubated in the labeling solution (0.313 mg protein/ml) at 37°C for 90 min. Fluorescence was measured on a Hitachi 850 fluorescence spectrophotometer with excitation and emission at 350 and 452 nm, respectively. Steady-state fluorescence anisotropy (r) was calculated according to the equation [13]:

$$r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH}) \quad (1)$$

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 [25, 49] and Lentz [24]. As pointed by Blitterswijk [51] and some investigators [13, 50], high degrees of fluorescence anisotropy indicate higher degrees of membrane order or lower degrees of membrane fluidity, and vice-versa.

Measurement of Lysosomal Membrane Potential

The recording of the membrane diffusion potential provides a means of probing the proton permeability of some membranes [31, 32]. The proton permeability of lysosomes can be assessed by measuring the membrane potential using carbocyanine dye $DiSC_3(5)$ as a probe [16, 56]. The assay medium contained 0.25 M sucrose, 0.5 μM $DiSC_3(5)$, buffered at pH 7.0 with 0.02 M Hepes/Tris. A 2.5–5 μl lysosomal sample was used for the assay. The fluorescence measurements were carried out at 25°C with excitation and emission wavelength of 622 and 670 nm, respectively, on a Hitachi 850 fluorescence spectrophotometer.

Table. Effects of BA and CHS treatments on the fluorescence anisotropy of DPH-labeled lysosomes

Treatment of lysosomes	Anisotropy (r)	P
Control[a]	0.157 ± 0.001	–
0.025 mM CHS 20 min[aa]	0.164 ± 0.001	<0.001
0.025 mM CHS 40 min[aa, b]	0.168 ± 0.001	<0.001
0.025 mM CHS 60 min[aa]	0.172 ± 0.004	<0.001
0.025 mM CHS 40 min, then 20 mM BA 15 min[bb]	0.160 ± 0.001	<0.001
Control[c]	0.159 ± 0.001	–
20 mM BA 7.5 min[cc]	0.152 ± 0.001	<0.001
20 mM BA 15 min[cc, d]	0.144 ± 0.002	<0.001
20 mM BA 30 min[cc]	0.139 ± 0.002	<0.001
20 mM BA 15 min, then 0.025 mM CHS 15 min[dd]	0.154 ± 0.001	<0.001

Lysosomal membranes were rigidified by the treatments with 0.025 mM CHS at 37°C for the indicated time. To increase the fluidity of the CHS-treated membranes (treatment for 40 min), the sample was treated subsequently with 20 mM BA for 15 min. For fluidizing the membranes, the lysosomes were incubated in the presence of 20 mM BA at 37°C for the indicated time. To decrease the fluidity of the BA-treated membranes (treatment for 15 min), the sample was treated subsequently with 0.025 mM CHS for 15 min. Lysosomal membrane fluidity was assessed by measuring DPH fluorescence anisotropy (r). All procedures were as described in Materials and Methods. Values are means ± SD, $n = 6$. Statistical analysis was performed using Student's t test. $P < 0.001$ compared to control values.
Note: *a*, *b*, *c* and *d* are the controls, *aa*, *bb*, *cc* and *dd*, respectively.

Measurement of Lysosomal Proton Leakage

Lysosomal proton leakage can acidify their suspending medium. The acidification of assay medium by the proton leakage was measured as previously described [57]. Briefly, a lysosomal sample was added to a 2 ml assay medium (contained 0.25 M sucrose and 0.1 mM p-nitrophenol) at 0.625 mg protein/ml, followed by the addition of 100 μl 0.75 M K_2SO_4. The absorbance (400 nm) of the pH sensitive dye p-nitrophenol was measured immediately after adding 10 μl 1 mM valinomycin to the medium. All measurements were carried out at 25°C on a Hitachi U-3200 spectrophotometer.

Abbreviations

The abbreviations used are: $DiSC_3(5)$, 3,3′-dipropylthiadicarbocyanine iodide; CCCP, carbonyl cyanide m-chlorophenylhydrazone; DPH, 1,6-diphenyl-1,3,5 hexatriene; BA, benzyl alcohol; CHS, cholesteryl hemisuccinate.

Results

Modulation of Lysosomal Membrane Fluidity

Lysosomal membrane fluidity was modulated by the treatments with membrane fluidizer BA and rigidifier CHS. As shown in the Table, the degree of fluorescence anisotropy (r) increased and decreased after the lysosomes were treated by CHS and BA, respectively. Treating the lysosomes with CHS for 20, 40 and 60 min increased the anisotropy (r) value by 4.46, 7.01 and 9.55%, respectively. In contrast, treatment of the lysosomes with BA for 7.5, 15 and 30 min decreased the anisotropy (r) value by 4.4, 9.43 and 12.58%, respectively. It indicates that the membrane order increased (or the membrane fluidity decreased) and decreased (or the membrane fluidity increased) by the treatments with CHS and BA, respectively. To decrease the fluidity of BA-treated lysosomal membranes, CHS was added to the incubation medium when the lysosomes were treated by BA for 15 min. After an additional 15 min incubation in the presence of CHS, fluorescence anisotropy (r) of the BA-treated lysosomes increased, showing that the BA-induced membrane fluidization was partly reversed by the subsequent treatment with CHS. As demonstrated by the results, rigidification of the CHS-treated lysosomal membranes (treatment of the lysosomes with CHS for 40 min) was partially reversed by the subsequent treatment with BA.

Effects of Lysosomal Membrane Fluidity on the Membrane Potential

Lysosomal proton permeability can be assessed by measuring their membrane potential using carbocyanine dye $DiSC_3(5)$ as a probe [16]. This positively charged dye accumulates within the lysosome which 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 is increased. Changes in the proton permeability of lysosomes by modulating their membrane fluidity were assessed using this method. As shown in Fig. 1*A*, the time order of BA treatment in decreasing the dye fluorescence of lysosomal samples is 15 min (line 3) > 7.5 min (line 2) > 0 min (line 1, control sample). Treating the lysosomes with BA for 7.5 and 15 min increased the fluorescence quenching by 4.07 and 7.55% (the difference of $\Delta F/F$ between BA-treated sample and control sample), respectively. The dye fluorescence did not decrease further with increasing the BA-treatment time from 15 to 30 min (*data not shown*), while the lysosomal membranes were further fluidized by continuing the BA treatment from 15 to 30 min (Table). The results suggest that the proton permeability of lysosomes increased with increases in a limited range of their membrane fluidity. The effect of the BA treatment on lysosomal proton permeability was further established by using protonophores in the fluorescence measurements. Since the protonophores such as CCCP and FCCP can make the lysosomal membrane permeable to protons and produce an equilibrium mem-

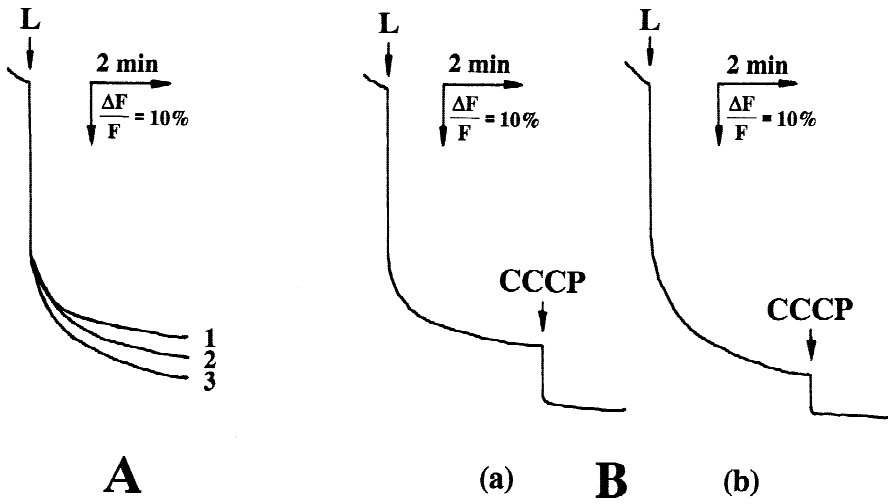

Fig. 1. Effects of BA treatment on lysosomal membrane potential. Procedures of the measurements of lysosomal membrane potential and treatments of lysosomes with BA were as described in Materials and Methods. Assay medium contained 0.25 M sucrose, 0.5 μM $DiSC_3(5)$, buffered at pH 7.0 with 0.02 M Hepes/Tris. Lysosomal sample (designated as 'L') and CCCP (2 μM) were added to the measuring medium at indicated time. Fluorescence of $DiSC_3(5)$ was measured at 25°C with excitation and emission wavelength of 622 and 670 nm, respectively. The fluorescence intensity is expressed as a percentage of its intensity just before addition of the lysosomes ($\Delta F/F$). Treatments of lysosomes with 20 mM BA for: (*A*), (1) 0 min (control), (2) 7.5 min, (3) 15 min; (*B*), (*a*) 0 min (control), (*b*) 15 min. A typical result out of three experiments is shown.

brane potential for the protons, an additional decrease in the fluorescence of $DiSC_3(5)$ can be produced by these agents during the measurement of lysosomal membrane potential. The total fluorescence quenching of the dye, i.e., the sum of the fluorescence quenching before adding protonophores and the quenching after adding protonophores, indicates the proton permeability of the membrane that is permeable to H^+ by the treatment with protonophores or the equilibrium membrane potential for the protons [16]. As shown in Fig. 1*B*, the degree of fluorescence decrease of the BA-treated lysosomal sample (line *b*) is larger than that of the control sample (line *a*) in the absence of CCCP, but the magnitude of CCCP-induced additional fluorescence quenching of the former is smaller than that of the latter. Although the extents of fluorescence quenching of these two samples are different either before or after adding CCCP, the total magnitudes of their fluorescence decrease are similar. The results indicate that the BA treatment did not alter the CCCP-produced lysosomal permeability to protons but increased the intrinsic proton permeability of the organelle and made it approach that induced by CCCP. Based on the results shown in the Table, the effect of BA treatment on the proton permeability of lysosomes is presumably due to the fluidization of their membranes. To examine whether this conclusion is correct, the fluidity of the BA-treated lysosomes was decreased by the subsequent CHS treatment described in the Table. Whether the increase in the proton permeability of the BA-treated lysosomes could be abolished by the CHS treatment was also examined. As shown in Fig. 2, the magnitude of the dye fluorescence quenching of BA-treated lysosomal sample (line 3), which is larger than that of control sample (line 2), is reduced by the subsequent CHS treatment (line 1). It indicates that the proton

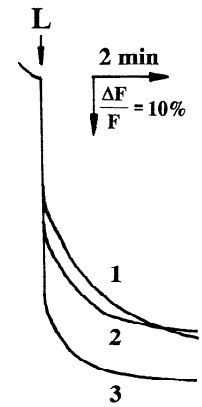

Fig. 2. Reversion of the effect of BA treatment on lysosomal membrane potential. Assay medium contained 0.25 M sucrose, 0.5 μM $DiSC_3(5)$, buffered at pH 7.0 with 0.02 M Hepes/Tris. Lysosomal sample (designated as 'L') was added to the measuring medium at indicated time. Fluorescence of $DiSC_3(5)$ was measured and expressed as described for Fig. 1. Treatments of lysosomes with: (1) 20 mM BA for 15 min, then 0.025 mM CHS for 15 min, (2) 20 mM BA for 0 min (control), (3) 20 mM BA for 15 min. Procedures of the treatments of lysosomes with BA and CHS were as described in Materials and Methods. A typical result out of three experiments is shown.

permeability of BA-treated lysosomes was reduced by the CHS treatment. The results support the conclusion that fluidizing the lysosomal membranes increased their proton permeability.

The effects of membrane rigidification on the lysosomal proton permeability was examined by comparing the proton permeability of CHS-treated lysosomes with that of control lysosomes. As shown in Fig. 3*A*, the time order of CHS treatment in decreasing the magnitudes of the dye fluorescence quenching of lysosomal samples is 40 min (line 1) > 20 min (line 2) > 0 min (line 3, control

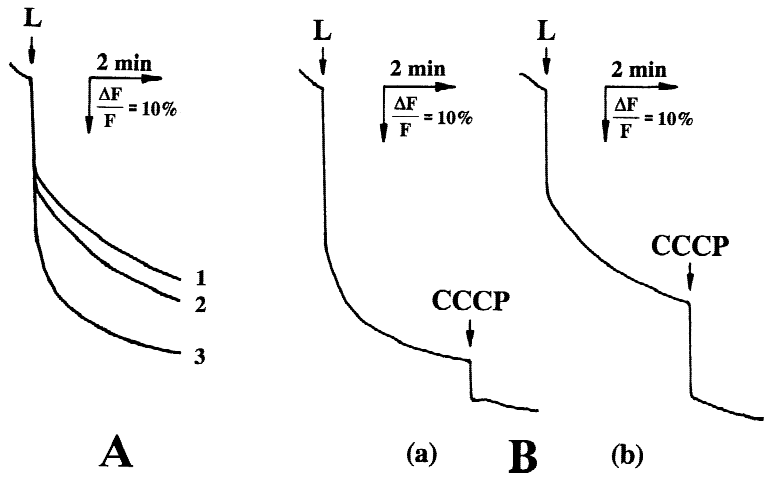

Fig. 3. Effects of CHS treatment on lysosomal membrane potential. Procedures of the measurements of lysosomal membrane potential and treatments of lysosomes with CHS were as described in Materials and Methods. Assay medium contained 0.25 M sucrose, 0.5 μM $DiSC_3(5)$, buffered at pH 7.0 with 0.02 M Hepes/Tris. Lysosomal sample (designated as 'L') and CCCP (2 μM) were added to the measuring medium at indicated time. Fluorescence of $DiSC_3(5)$ was measured and expressed as described for Fig. 1. Treatments of lysosomes with 0.025 mM CHS for: (*A*), (1) 40 min, (2) 20 min, (3) 0 min (control); (*B*), (*a*) 0 min (control), (*b*) 40 min. A typical result out of three experiments is shown.

sample). Treating the lysosomes with CHS for 20 and 40 min decreased the fluorescence quenching by 9.24 and 13.42% (the difference of $\Delta F/F$ between the CHS-treated sample and the control sample), respectively. The degree of the dye fluorescence quenching did not decrease further when the CHS-treatment time was increased from 40 to 60 min (*data not shown*) although the lysosomal membrane fluidity could decrease further with continuing the CHS treatment from 40 to 60 min (Table). The results suggest that the proton permeability of lysosomes decreased with the decreases in a limited range of their membrane fluidity. The effect of the CHS treatment on lysosomal proton permeability was further established by the data shown in Fig. 3*B* (*cf.* explanation of Fig. 1*B*). Given that the CHS treatment can rigidify lysosomal membranes, the decrease in proton permeability of the CHS-treated lysosomes is presumed to be due to the rigidification of their membranes. To confirm this conclusion, we attempted to determine if the decrease in proton permeability of the CHS-treated lysosomes could be abolished by fluidizing their membranes. The results show that the magnitude of decrease in the dye fluorescence of CHS-treated lysosomal sample (Fig. 4, line 1), which is smaller than that of control sample (line 3), was increased by the subsequent BA treatment (line 2). The reversion of the CHS treatment-induced decrease in lysosomal proton permeability by the BA treatment is presumably due to the fluidizing effect of BA on the CHS-treated membranes (Table). These results provide strong evidence that rigidifying lysosomal membranes decreased their proton permeability.

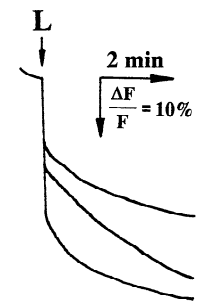

Fig. 4. Reversion of the effect of CHS treatment on lysosomal membrane potential. Assay medium contained 0.25 M sucrose, 2.5 μM $DiSC_3(5)$, buffered at pH 7.0 with 0.02 M Hepes/Tris. Lysosomal sample (designated as 'L') was added to the measuring medium at indicated time. Fluorescence of $DiSC_3(5)$ was measured and expressed as described for Fig. 1. Treatments of lysosomes with: (1) 0.025 mM CHS for 40 min, (2) 0.025 mM CHS for 40 min, then 20 mM BA for 15 min, (3) 0.025 mM CHS for 0 min (control). Procedures of the treatments of lysosomes with CHS and BA were as described in Materials and Methods. A typical result out of three experiments is shown.

Effects of Lysosomal Membrane Fluidity on the Proton Leakage

Since ion flux through a membrane is proportional to the ion permeability [6], the lysosomal proton permeability can be assessed by measuring their proton leakage-induced acidification of the suspending medium with the pH-sensitive dye p-nitrophenol as an indicator [57]. The measurements were carried out by monitoring the decrease in p-nitrophenol absorbance at 400 nm, which were based on the property of the dye that the unprotonated p-nitrophenol molecules have a sufficiently larger extinction coefficient at 400 nm over that of protonated molecules [27]. To examine the effect of lysosomal proton leakage on the absorbance of p-nitrophenol, the measurement was performed upon addition of CCCP and valinomycin to a K^+-containing measuring medium. As shown in Fig. 5*a*, absorbance of the dye was greatly reduced in the presence of the ionophores (line 5), presumably due to the proton leakage induced by an exchange of intralysosomal H^+ for external K^+. This decreasing effect of proton leakage on the dye absorbance was confirmed by the observation that the absorbance

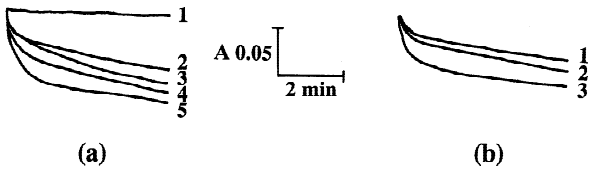

Fig. 5. Effects of BA and CHS treatments on lysosomal proton leakage. Lysosomal sample was added to a 2 ml assay medium (contained 0.25 M sucrose and 0.1 mM p-nitrophenol) at 0.625 mg protein/ml, followed by the addition of 100 μl 0.75M K_2SO_4. Absorbance (400 nm) of p-nitrophenol was measured immediately after adding 10 μl 1mM valinomycin to the medium. (*a*) Treatments of lysosomes with 20 mM BA for: (1) 15 min, assay medium pH was maintained at 6.0 with 0.1 M citrate buffer, (2) 0 min (control), (3) 7.5 min, (4) 15 min, (5) 0 min (control), assay medium contained 1 μM CCCP; (*b*) Treatments of lysosomes with 0.025 mM CHS for: (1) 40 min, (2) 20 min, (3) 0 min (control). Procedures of the treatments of lysosomes with BA and CHS were as described in Materials and Methods. A typical result out of three experiments is shown.

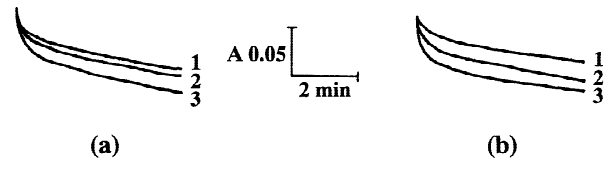

Fig. 6. Reversion of the effects of BA and CHS treatments on lysosomal proton leakage. Lysosomal sample was added to a 2 ml assay medium (contained 0.25 M sucrose and 0.1 mM p-nitrophenol) at 0.625 mg protein/ml, followed by the addition of 100 μl 0.75 M K_2SO_4. Absorbance (400 nm) of p-nitrophenol was measured immediately after adding 10 μl 1 mM valinomycin to the medium. (*a*) Treatments of lysosomes with: (1) 20 mM BA for 0 min (control), (2) 20 mM BA for 15 min, then 0.025 mM CHS for 15 min, (3) 20 mM BA for 15 min; (*b*) Treatments of lysosomes with: (1) 0.025 mM CHS for 40 min, (2) 0.025 mM CHS for 40 min, then 20 mM BA for 15 min, (3) 0.025 mM CHS for 0 min (control). Procedures of the treatments of lysosomes with BA and CHS were as described in Materials and Methods. A typical result out of three experiments is shown.

decrease could be abolished by buffering the measuring medium (*data not shown*). As indicated by the results shown in Figs. 1–4, changes in the lysosomal membrane fluidity affect their proton permeability. This conclusion was reexamined by measuring proton leakage of the BA- and/or CHS-treated lysosomes using p-nitrophenol as an indicator. The dye absorbance of the lysosomal samples treated by BA for 7.5 and 15 min (Fig. 5*a*, lines 3 and 4) and the control sample measured in the presence of CCCP (line 5) reduced by 125, 142 and 158% of the decrease in absorbance of the control sample (line 2), respectively. Since the dye absorbance is inversely proportional to the proton leakage in the conditions of this study (*data not shown*) and the proton efflux is proportional to the proton permeability, the results indicate that the BA treatment (15 min) increased the proton permeability by 42%, which approached the increase in the proton permeability (58%) induced by protonophore CCCP. Although the absorbance reduced with increasing the BA-treatment time from 0 min to 15 min (lines 2, 3 and 4), it did not decrease further when the BA-treatment time increased from 15 to 30 min (*data not shown*). In addition, the decrease in absorbance of the sample treated by BA for 15 min could be abolished by buffering the measuring medium (line 1). The results indicate that the decrease in the dye absorbance of BA-treated samples was due to a proton leakage-induced acidification of the lysosomal suspension and suggest that the proton permeability of lysosomes increased with the increases in a limited range of their membrane fluidity. In contrast to the effect of the BA treatment, treating the lysosomes with CHS decreased their proton leakage. As shown in Fig. 5*b*, the degrees of decrease in the dye absorbance of lysosomal samples reduced with increasing the CHS-treatment time from 0 to 40 min (lines 3, 2 and 1). The absorbance decrease of the lysosomal samples treated by CHS for 20 and 40 min (lines 2 and 1) is 77.14 and 61.91% of the decrease in absorbance of the control sample (line 3), respectively. It indicates that treatments of lysosomes with CHS for 40 min decreased the proton permeability by 38.1%. The extent of the absorbance decrease did not reduce further with increasing the CHS-treatment time from 40 to 60 min (*data not shown*), suggesting that the proton permeability of lysosomes decreased with the decreases in a limited range of their membrane fluidity.

As demonstrated above, the lysosomal proton leakage increased and decreased after increasing the membrane fluidity with BA and decreasing the membrane fluidity with CHS, respectively. To examine if the changes in the lysosomal proton leakage were due to the alterations in their membrane fluidity, the membrane fluidity of the BA and CHS treated lysosomes was decreased and increased, respectively, and the resulted variations in their proton leakage were assessed. As shown in Fig. 6*a*, treating the lysosomes with BA for 15 min caused an additional decrease in the dye absorbance in comparison with the absorbance decrease of control lysosomes (compare line 3 with line 1). The magnitude of the absorbance decrease of the BA-treated lysosomal sample reduced markedly after the lysosomes were treated subsequently by CHS (line 2). It indicates that treating the lysosomes with BA increased their proton leakage and that the CHS treatment decreased the proton leakage of BA-treated lysosomes. The results, shown in Fig. 6*b*, demonstrate that the CHS treatment decreased the lysosomal proton leakage (compare line 1 with line 3) and that fluidizing the membranes of the CHS-treated lysosomes with BA increased their proton leakage (compare line 2 with line 1). Thus, it was established that changes in the proton leakage of the BA and CHS treated lysosomes were induced by the alterations in their membrane fluidity.

Taken together, the above results of the assessments

of lysosomal membrane potential and proton leakage suggest that the proton permeability of lysosomes can be modulated by the changes in a limited range of their membrane fluidity.

Discussion

The membrane lipid fluidity can be modulated by a wide range of physiological variables such as fatty acid composition [22], aging [33], alcohols [10], phosphatidylethanol [38], sterols [46], insecticides [2], diacylglycerols [39], drug-induced cytochrome P-450 activity [20] and ether lipids [19]. On the other hand, a variety of membrane physiological and biochemical properties such as membrane permeability and membrane-bound enzyme activity can be regulated by the changes in the membrane fluidity [43, 47]. Since the mutual regulations between membrane lipid fluidity and cellular activities are important for cell life, clarifying the influence of various factors on the membrane fluidity, the effects of membrane fluidity on the membrane activities, and the related mechanisms continues to be an active area of investigation. As pointed out by Girotti [14], the lipid peroxidation of membranes is generally linked to the loss of their fluidity. A number of studies demonstrated that lysosomal membrane lipid is susceptible to oxidation [29, 59] and the oxidation-induced membrane lipid rigidification [59]. Since free radicals can be produced in vivo, the lysosomal membranes may be affected by the oxidative environment [29]. In addition to membrane lipid peroxidation, accumulation of polyanions in lysosomes can also rigidify their membranes [21]. It has recently been established that some bioactive compounds such as bilirubin, farmorubicin and chelerythrine can modulate the membrane fluidity of lysosomes [35]. In addition, lysosomal membrane fluidity may change in apoptosis [48]. All these evidences suggest that lysosomal membrane fluidity is liable to change under various conditions. As described above, a variety of lysosomal activities can be affected by their proton permeability, but what nature and/or component of lysosomal membranes influence the property is still unknown. For some membranes, changes in the membrane fluidity affect their proton permeability [3, 42, 43]. Whether the membrane fluidity of lysosomes influences their proton permeability is still unclear. To elucidate this issue may have some pathophysiological significance in the studies of lysosomes.

In this work, the influence of membrane fluidity on the lysosomal permeability to H^+ was studied by fluidizing and rigidifying the membranes with BA and CHS, respectively. As a result of the treatments with these agents, the lysosomal proton permeability changed. It is consistent with the results of previous studies [3, 42, 43], in which the proton permeability of some membranes varied after their fluidity was modulated by either modifying the membrane cholesterol content or altering the membrane incubation temperature. As demonstrated by these studies and the present work, the increases or decreases in membrane fluidity correspond to the increases or decreases in their proton permeability. Although the precise mechanism for such a correlation is still unclear, a plausible explanation has been provided. As suggested by Deamer and Nichols [7], the proton permeation correlates to the physical properties of the membranes and involves transient formation of hydrogen bonded chains of water within the membranes. A small fraction of the water in the bilayer might be associated through hydrogen bonding, thereby providing a conductance pathway unique to protons. By now, at least four pathways or mechanisms for the water flux across membranes have been proposed, including aqueous pore [11], solubility-diffusion mechanism [11], channel [28] and transient defects in the membrane arising from thermal fluctuation [6]. The permeation of protons across membranes are suggested as being along the hydrogen bonds of water which crosses the membranes through aqueous pores [30] and transient defects [6]. The physical state of the lipid bilayer has been shown to be an important determinant of water permeability [52]. This view was supported by various lines of evidence that the water permeability of some membranes can be increased and decreased by increasing and decreasing their membrane fluidity, respectively [4, 54, 55]. It has been established that fluidizing or rigidifying erythrocyte membranes can increase or decrease the size of their aqueous pores, respectively [44]. In addition, the transient defects of membranes can be increased in number by the temperature-dependent phase transitions and membrane perturbants [6]. These descriptions and results suggest that changes in the membrane fluidity may alter the water permeability and therefore affect the proton permeability. It should be noted that a number of studies find little correlation between membrane fluidity and proton permeability for some membranes [18, 23, 45, 53]. In the rabbit renal brush-border membranes, the permeation of protons is not correlated with the water permeability [53]. It suggests that the effects of membrane fluidity and water permeability on the proton permeation are dependent on the membrane types. By now, little information is available concerning the pathways for the water flux across lysosomal membranes. How water flow across lysosomal membranes and why membrane fluidity can affect lysosomal proton permeability remain for further study.

To estimate the maximum proton permeability, we permeablized the lysosomal membranes to protons using protonophore CCCP. The results show that treating the

lysosomes with CCCP increased the proton permeability only by 58% (Fig. 5*a*). As demonstrated previously [16], treatment with protonophore FCCP slightly increased lysosomal membrane potential, which reflects a slight increase in the proton permeability [16]. It suggests that lysosomal membrane exhibits a significant permeability to protons [16, 36]. Compared with CCCP, the BA treatment (15 min) increased the proton permeability by 42%. In contrast, the CHS treatment (40 min) decreased the proton permeability by 38.1%. It indicates that the physical state of lysosomal membranes can affect their proton permeability.

As pointed out by Reeves [41], the K^+-specific ionophore valinomycin will allow external K^+ to enter the lysosome at a rapid rate, thus providing a charge-compensating mechanism that will permit protons to diffuse out of the lysosome at a rate determined solely by the intrinsic permeability of the membrane toward H^+. Without valinomycin, the protonophore CCCP has only minor effects on the lysosomal pH, presumably because H^+ efflux is limited by the absence of permeable counterions. Similar description was also provided by Dell'Antone [8]. In this study, the proton permeability of lysosomes was assessed by measuring their proton leakage. Although the proton permeability of normal lysosomes differs from that of the membrane fluidity-modulated ones, it is difficult to detect the differences in their intrinsic proton leakage in a sucrose medium. According to the concept described by Reeves and Dell'Antone, we detected the differences in proton leakage of the lysosomes exhibiting different membrane fluidity upon addition of valinomycin and K_2SO_4 to the measuring medium.

Previous studies measured lysosomal proton permeability indirectly either by the osmotic protection method [5] or by the assessment of intralysosomal pH [17]. In the present work, we measured the lysosomal membrane potential to estimate the proton permeability by using of the fluorescence dye $DiSC_3(5)$. This method used by Harikumar and Reeves [16] is based on the property of the dye that the redistribution of the positively charged dye from the outside medium into the lysosomes, which causes fluorescence quenching of the dye, depends on the negative internal potential [12]. As shown in their study [16], the lysosomes are polarized inside negatively due to the pH gradient across the membranes and increases in the proton permeability can cause the membrane potential to become more negative inside. Thus, the lysosomal proton permeability can be estimated indirectly by measuring the dye fluorescence.

The results of this study show that the membrane physical state of lysosomes influences their permeability to protons. It suggests that the lysosomal proton permeability in living cells might be affected by the fluidity-modulating factors.

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