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Involvement of a non-proton pump factor (possibly Donnan-type equilibrium) in maintenance of an acidic pH in lysosomes

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Change of the internal pH of isolated lysosomes was measured with fluorescein isothiocyanate–dextran. In buffer of pH 7.0, isolated lysosomes had an acidic pH of about 5.5, which decreased to pH 5.2 on addition of ATP. Addition of bafilomycin inhibited the acidification by H^+-ATPase and resulted in an increase of the internal pH to 5.5 due to passive diffusion of protons across the lysosomal membrane. However, no further alkalization was observed. The acidic pH (pH 5.5) of isolated lysosomes could be maintained for at least 48 h in the absence of ATP, but increased gradually to pH 5.9–6.4 upon incubation with monovalent cations (K^+ or Na^+), amines, or ionophores. These results suggest that a non-proton pump factor (possibly Donnan equilibrium) is involved in maintaining the acidic pH of isolated lysosomes.

Lysosome; Acidic pH; Proton pump; Donnan equilibrium

1. INTRODUCTION

An acidic internal pH is an intrinsic property of lysosomes, secretory granules and related organelles [1–3]. As an ATP-dependent proton pump (vacuolar-type H^+-ATPase) has been found in these membranes [4,5], proton transport by this enzyme is generally thought to be the sole mechanism for establishing and maintaining the acidic internal pH of the organelles [1,2]. However, this H^+-ATPase may not be the only component responsible for the pH homeostasis in lysosomes, because lysosomes maintain an acidic pH after isolation in the absence of ATP [4]. It has been unknown how long and by what mechanism this pH is maintained.

As reported here, we found that the acidic pH of isolated lysosomes could be maintained in buffered sucrose without active influx of protons and that the acidity could be abolished by cations such as K^+ or Na^+. The mechanism of maintenance of the acidity by non-proton pump factor is discussed.

2. MATERIALS AND METHODS

2.1. *Preparation of lysosomes*

Lysosomes with entrapped fluorescein isothiocyanate–dextran (FITC-dex) was prepared as follows: FITC-dex (20 mg/ml, 1 ml) was injected intraperitoneally into male ddY strain mice weighing about 35 g. After overnight starvation, the mice were decapitated and their livers were isolated and suspended in 0.3 M sucrose (pH 7.0) containing 0.1% ethanol and 1 mM EDTA. Lysosomes were then prepared by differential centrifugation and suspended in the same solution [4,6]. Triton-filled lysosomes (tritosomes) were prepared as follows [4,6]: Triton WR-1339 (0.1 mg/ml, 1 ml) was injected into mice intraperitoneally. Three days later, the livers were isolated, and homogenized in 0.3 M sucrose, 0.1% ethanol and 1 mM EDTA (pH 7.0), and the lysosome-rich fraction was prepared. The lysosomes were purified by sucrose density gradient centrifugation and used on the day of preparation.

Abbreviations: FITC-dex, fluorescein isothiocyanate dextran; MOPS, morpholinopropane sulfonic acid.

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2.2. *Measurement of intra-lysosomal pH*

The intra-lysosomal pH was monitored by measuring FITC-dex (FD-70S; Sigma) fluorescence [4,6]. The lysosomal fraction (90 μg protein) was incubated in 20 mM MOPS-Tris (pH 7.0) containing 0.3 M sucrose, 0.1 M KCl and 2 mM $MgCl_2$, and fluorescence was measured in a Hitachi F6000 spectrofluorometer at 25°C with excitation and emission wavelengths of 495 and 550 nm, respectively.

For quantitative estimation of the intra-lysosomal pH, the ratio of FITC-dex fluorescence intensities at 550 nm with excitations at 495 nm (pH-sensitive fluorescence) and 450 nm (pH-insensitive fluorescence) was determined after subtracting the background fluorescence (mainly due to light scattering and extra-lysosomal FITC-dex):

$$\text{Ratio} = \frac{F_{(550\text{ nm observed})} - F_{(550\text{ nm background})}}{F_{(450\text{ nm observed})} - F_{(450\text{ nm background})}}$$

A standard curve was prepared by measuring the fluorescence of 2 μg of FITC-dex in buffers of different pH values. Another standard curve was made by suspending lysosomes with FITC-dex entrapped in vivo in buffers of different pH values and disrupting them with 0.1% Triton X-100. These two standard curves were essentially the same.

2.3. *Measurement of membrane potential*

The membrane potential of lysosomes was measured fluorometrically with diS-C3(5) as a fluorescent membrane potential probe [6]. The standard assay solution contained 2 ml of 20 mM MOPS-Tris (pH 7.0) containing 0.5 M sucrose, 2 mM $MgCl_2$ and 1 μM diS-C3(5). The excitation and emission wavelength pair used was 620 and 670 nm.

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2.4. *Other procedures*

The latency of the lysosomes was estimated by measuring *N*-acetyl-β-D-glucuronidase or acid phosphatase in the presence or absence of Triton X-110 (0.05%) [7]. Proteins were measured by the method of Bradford [8].

3. RESULTS AND DISCUSSION

FITX-dex was entrapped in liver lysosomes after its intraperitoneal injection into mice, and its fluorescence allowed continuous measurement of the pH inside isolated lysosomes [4,6]. Addition of ATP induced quenching of the fluorescence, indicating lysosomal acidification by H^+-ATPase (Fig. 1A), whereas addition of nigericin (1 μM) increased the fluorescence, indicating lysosomal alkalinization due to H^+/K^+ exchange induced by the ionophore (Fig. 1B). Quantitatively, the internal pH of isolated lysosomes was about 5.5, decreased to 5.2 with ATP, and increased to about 6.3 with nigericin. No further acidification by H^+-ATPase was observed, possibly because of equilibrium of passive efflux of protons and ATP-driven influx of protons. The lysosomal H^+-ATPase may also be controlled by the membrane potential, as observed with other vacuolar H^+-ATPases [9].

We found that bafilomycin, a specific inhibitor of vacuolar H^+-ATPase [10], completely inhibited the ATP-dependent acidification, but had no effect on the pre-established acidic pH (pH 5.5) (Fig. 1C, E). When the same antibiotic was added after addition of ATP, the internal pH returned to 5.5 due to passive efflux of protons. However, the lysosomal pH did not increase further to the pH observed after addition of nigericin: pH 5.5, with bafilomycin (Fig. 1F), pH 6.3, with nigericin (Fig.1B). These results indicated that the acidic pH of isolated lysosomes (pH 5.5) was resistant to passive efflux of protons and could be maintained without active uptake of protons by H^+-ATPase.

The acidic pH (pH 5.5) of lysosomes in the isolation buffer (pH 7.0) was maintained for at least 2 days at 4°C even in the absence of ATP (Fig. 2A). After prolonged storage, significant disruption of the lysosomes occurred and so that intra-lysosomal pH could not be measured precisely. The presence of bafilomycin (10 μM) and addition of hexokinase and glucose (to remove endogenous ATP) during the incubation did not affect the acidic pH at all, again indicating that this acidic pH was independent of H^+-ATPase.

To determine how the acidic pH was maintained without active influx of protons, we tested the effects of various salts on the lysosomal pH, because ion equilibrium has been suggested to participate in pH homeostasis in lysosomes [2,3]. The additions of NaCl and KCl at 0.1 M resultes in a gradual increase in the internal pH to 6.0 (Fig. 2B). Monovalent cations were responsible for this alkalinization, their order of effectiveness being K^+, Rb^+ > Na^+ ≫ $choline^+$, while anions including Cl^-,

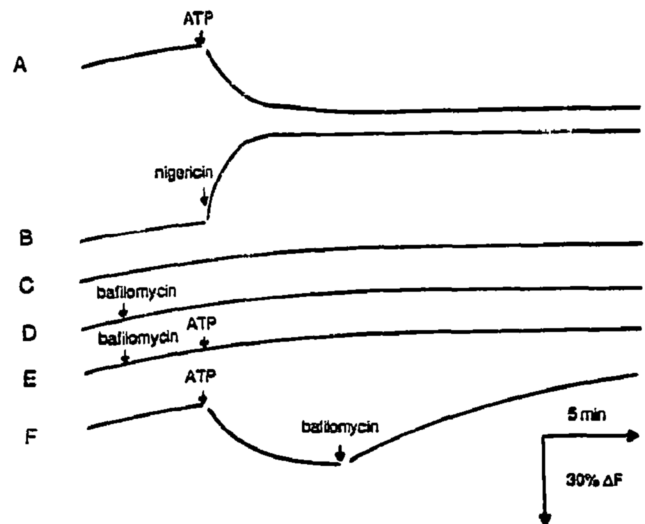

Fig. 1. Measurement of intralysosomal pH and the effect of bafilomycin. Change of lysosomal pH was monitored as that of FITC-dex fluorescence. At the times indicated by arrows, ATP, nigericin and bafilomycin were added at final concentrations of 0.5 mM, 1 μM, and 0.1 μM, respectively. Essentially the same results were obtained with highly purified lysosomes loaded with Triton WR1339.

Br^-, SCN^-, SO_4^{2-} and PO_4^{2-} and divalent cations (Mg^{2+}, Ca^{2+}) had no effect (not shown). More rapid dissipation of the pre-established acidic pH could be attained with ammonium ion (Fig. 2A), various amines and ionophores (cation/proton exchangers), which increased the internal pH to 6.4 (Table I). These results indicated that most of the acidic pH could be dissipated by increasing cation permeability, and suggested the existence of localized H^+ in Donnan-type ion equilibrium which could be replaced by other cations.

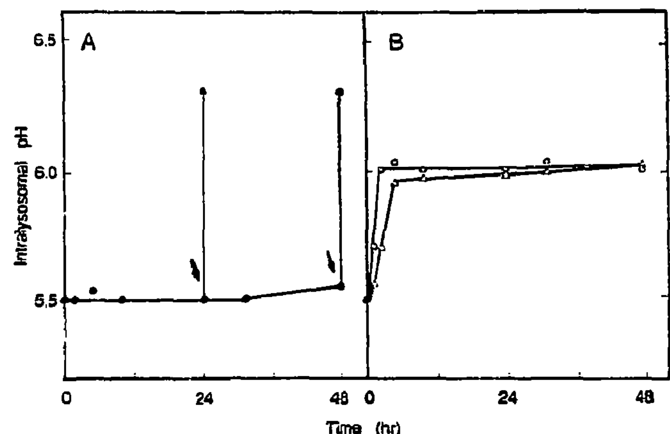

Fig. 2. Stability of acidic pH in isolated lysosomes. Lysosomes were isolated, suspended in 20 mM MOPS-Tris (pH 7.0) containing 0.3 M sucrose and 1 mM EGTA (●) (Fig. 2A) or in the same buffer with 0.1 M KCl (○) or NaCl (△) (Fig. 2B), and incubated in an ice bath. At the indicated times, aliquots (20 μl) were taken and the fluorescence of FITC-dex in the steady-state was measured. Ammonium chloride (10 mM) was added at the times indicated by arrows, and the pH was promptly determined.

Table I
Effects of amines and ionophores on lysosomal pH

	Internal pH
No addition	5.5
NH_4Cl (10 mM)	6.4
Chloroquine (0.1 mM)	6.4
Nigericin (2 μM)	6.3
Nigericin (2 μM) + NH_4Cl (10 mM)	6.4
Monensin (5 μM) + NaCl (0.1 M)	6.2
Gramicidin (10 μM)	5.9

The internal pH of lysosomes in the presence of the listed compounds was measured as described in the legends of Fig. 1 and 2.

Lysosomes are known to contain negatively charged molecules, possibly in membrane-impermeable forms [11,12] and are negatively polarized inside [2,6]. These negative charges could form a Donnan potential and maintain the acidic internal pH by preventing free diffusion of protons. It is noteworthy that the membrane potential was also insensitive to bafilomycin and could be maintained without ATP (not shown). Furthermore monovalent cations could depolarize the membrane potential and their order of effectiveness was the same as that for alkalinization listed above [6]. These properties were consistent with the idea of the existence of Donnan-type equilibrium in lysosomes.

From these findings, we propose the following scheme for the mechanism of formation and maintenance of the acidic pH in lysosomes: (i) protons are transported into lysosomes by H^+-ATPase (primary supply of protons); (ii) then, negatively charged molecules in the lysosomes reach Donnan-type equilibrium with the protons and maintain a stable acidic pH. This acidic pH could be maintained without further proton transport by H^+-ATPase, unless monovalent cations were added externally. External monovalent cations exchange with protons inside the lysosomes (Fig. 2), and the resulting elevated pH could be decreased again to pH 5.3 by H^+-ATPase. Bafilomycin raises the intralysosomal pH to about 6.5 in living cells [13], although it did not increase the pH in isolated lysosomes incubated in the absence of monovalent cations. Thus, the lysosomal pH in vivo may be due to the dynamic equilibrium between influx of protons by H^+-ATPase, their efflux in exchange with monovalent cations in the cytoplasm and Donnan equilibrium.

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