MEMBRANOUS ATPASES AND TRANSPORT FUNCTIONS OF LYSOSOMES

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A conspicuous feature of lysosomes is that their interior is acidic providing favorable conditions for acid hydrolases therein. Its precise pH as well as its maintenance mechanism have long been a matter of dispute. We estimated the intralysosomal pH using fluorescein isothiocyanate-dextran (FITC-dextran, FD) fluorescence to be slightly lower than 5.0 and showed that it is maintained using metabolic energy (12). We also showed that the intralysosomal acidity is raised by exposure of cells to weak basic substances or acidic ionophores (12, 19, 21). These findings have attracted attention from a variety of fields, because these chemicals have been known to affect a variety of biologically important phenomena. The interest in the physiological significance of this phenomenon has been strengthened furthermore by the recent discovery of additional acidic pockets, namely, endosomes, Golgi apparatus, secretory granules, kidney plasmalemmal vesicles, etc. (2, 16, 17).

I. PROPERTIES OF LYSOSOMAL PROTON PUMP

By using FD as a sensitive fluorescent pH probe, we identified ATP-dependent proton pump (H^+ -pump) on lysosomal membranes, which functions to transport protons into lysosomes (14, 15). The lysosomal proton pump is unique both in its sensitivity to NEM, NBD-Cl, quercetin, ADP, AMP-PNP and DCCD and in its insensitivity to oligomycin, vanadate and ouabain. Table 1 summarizes the characteristics of the proton pump on rat liver lysosomes.

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Abbreviations used: AMP-CPP: α , β -methylene-ATP; AMP-PCP: β , γ -methylene-ATP; AMP-PNP: adenylyl-imidodiphosphate, ATP: adenosine-5'-triphosphate, DCCD: *N*, *N'*-dicyclohexylcarbodiimide, DES: diethylstilbestrol, DIDS: 4, 4'-diisothiocyanatostilbene-2, 2'-disulphonic acid, FD: fluorescein isothiocyanate-dextran, FCCP: carbonylcyanide *p*-trifluoromethoxyphenylhydrazone, IC_{50} : concentration for 50% of maximum inhibition, NBD-Cl: 7-Chloro-4-nitrobenzo-2-oxa-1, 3-diazole, NEM: *N*-ethylmaleimide, PCMB: *p*-chloromercuribenzoic acid, SITS: 4-acetoamide-4'-isothiocyanatostilbene-2, 2'-disulfonic acid.

TABLE 1. *Properties of lysosomal proton pump as revealed with fluorescein-isothiocyanate dextran fluorescence.*

Substrate sepecificity:	
effective	ATP=dATP=(>*) GTP, ITP>>>CTP, UTP
ineffective	PNPP, AMP, AMP-CPP, AMP-PCP
inhibitory	ADP, AMP-PNP
K _m for ATP:	≈0.1 mM
Generation of membrane potential	electrogenic
Monovalent cation requirement	not detected
Divalent cation requirement:	
effective	Mg ²⁺ , Mn ²⁺
ineffective	Fe ²⁺ , Ba ²⁺ , Ca ²⁺ , Ba ²⁺ , Sr ²⁺
inhibitory	Cu ²⁺ (IC ₁₀₀ ≈100 μM), Zn ²⁺ (IC ₁₀₀ ≈100 μM), Hg ²⁺ , Cd ²⁺
Anion requirement:	membrane-permeant anion is required for acidification (Cl ⁻ (K _m of transport: ≈3 mM), Br ⁻ > phosphate, SO ₄ ²⁻ , F ⁻)
Sensitivity to ATPase inhibitors:	
sensitive	DCCD (IC ₁₀₀ ≈25 μM), tri- <i>n</i> -butyltin (IC ₁₀₀ ≈25 μM), SH-reagents [NEM (IC ₅₀ =50 μM, IC ₁₀₀ =0.5 mM), PCMB (IC ₅₀ <1 μM)], phenylisothiocyanate (IC ₁₀₀ ≈200 μM), tri-nitrophenylsulphonate (IC ₁₀₀ ≈250 μM), NBD-Cl (IC ₁₀₀ ≈25 μM), quercetin (IC ₁₀₀ ≈25 μM), stilbene derivatives [SITS (IC ₁₀₀ ≈2.5 μM), DIDS (IC ₁₀₀ ≈10 μM), DES (IC ₁₀₀ ≤10 μM)], bafilomycin (IC ₅₀ ≈0.9 nM), pyridoxal 5-phosphate (IC ₁₀₀ ≈0.75 mM), diethylpyrocarbonate (IC ₁₀₀ ≈60 μM), phenylglyoxal (IC ₁₀₀ ≈1 mM), 2, 3-butandione (IC ₁₀₀ ≈11.5 mM), <i>N</i> -bromosuccinimide (IC ₁₀₀ ≈0.6 μM), dibromoacetophenone (IC ₁₀₀ ≈0.3 μM)
partially sensitive	NaN ₃ (≤30% inhibition at 10 mM)
insensitive	ouabain, vanadate, oligomycin, efrapeptin

* rat liver tritosomes

II. PROPERTIES OF ENDOSOMAL PROTON PUMP

Since our discovery of the lysosomal proton pump, a number of papers have appeared on proton pump activities on various membranes of the vacuolar system (1, 20). Fig. 1 shows some of our results on the properties of the proton pump on endosomes (late endosomes) isolated from cultured mouse peritoneal macrophages. Its characteristics are quite similar to those of the lysosomal proton pump.

III. PROPERTIES OF LYSOSOMAL ATPASES

We showed that alkaline Mg²⁺-ATPase activity is associated with the membrane ghosts derived from rat liver lysosomes. Its characteristics show some similarity with those of the proton pump (10). Other unpublished characteristics are as follows: It is

Fig. 1. Selected properties of endosomal proton pump as revealed with FD fluorescence. A) substrate specificity, B) Divalent cation requirement, C) anion requirement, D) inhibitor sensitivity. Late endosome fractions containing FD were obtained from cultured mouse peritoneal macrophages and separated on Percoll density gradient centrifugation and its proton pump activity was determined fluorometrically (20). Nucleotides, 1 mM; FCCP, 2.5 μ M; metals, 1 mM as chloride; salts, 0.1 M; sucrose, 0.3 M; NEM, 1 mM; oligomycin, 5 μ M; DCCD, 25 μ M; NaVO_3 , 5 mM; DIDS, 50 μ M; PCMB, 10 μ M; tributyltin, 5 μ M; NBD-Cl, 100 μ M.

slightly accelerated by FCCP and nigericin but not by valinomycin, sensitive to phenylglyoxal (maximum 26% inhibition at 8 mM), *N*-bromosuccinimide ($\text{IC}_{50} = 0.22$ mM), diethylpyrocarbonate ($\text{IC}_{50} = 0.22$ mM), dibromoacetophenone (maximum 26% inhibition at 1 mM), tetracaine ($\text{IC}_{50} = 4.0$ mM), but insensitive to pyridoxal phosphate.

However, there are some differences between the proton pump and ATPase in sensitivity to chemicals: namely, in contrast to proton pump activity, ATPase activity is only marginally sensitive to SH-reagents [NEM (maximum $\approx 20\%$ inhibition at 1 mM with $\text{IC}_{50}' = 0.1$ mM) or PCMB (no inhibition)] and bafilomycin (maximum $\approx 10\%$ inhibition with $\text{IC}_{50}' = 10$ nM), and almost insensitive to NBD-Cl, quercetin, stilbene derivatives [SITS, DIDS, DES, dienestrol, hexestrol] and pyridoxal phosphate. Furthermore, ATPase activity is expressed even with Ca^{2+} as divalent cation.

Such being the case, we looked into the properties of ATPase activities of other sources of lysosomes, namely rat kidney. Fig. 2 shows selected properties of Mg^{2+} -ATPase on rat kidney lysosomes. Kidney ATPase activity showed the pH profile

FIG. 2. Selected properties of ATPase activity on lysosomal membrane ghosts of rat kidney. A) pH profile in the presence (■) and absence (□) of 2 mM MgCl₂. B) Inhibitor sensitivity of lysosomal (●) as compared with rat kidney submitochondrial particle (○) ATPase. Rat kidney lysosomes and their membrane ghosts were prepared according to (8) and (10).

(Fig. 2A), the nucleotide specificity (ATP : 2-deoxy-ATP : GTP : ITP : UTP : CTP : ADP : AMP : AMP-PNP = 100 : 83 : 56 : 56 : 28 : 27 : 35 : 6 : 8) and the divalent cation specificity (Mg^{2+} : Mn^{2+} : Co^{2+} : Ca^{2+} : Zn^{2+} : Cd^{2+} : none = 100 : 74 : 18 : 14 : 6 : 3 : 3) similar to those of liver enzyme. Monovalent cation requirement was not detected. However, in contrast to liver enzyme, it was highly sensitive to NEM (maximum 70% inhibition with $\text{IC}_{50}' = 4 \mu\text{M}$), PCMB (maximum 50% inhibition with $\text{IC}_{50}' = 0.5 \mu\text{M}$), DCCD, NBD-Cl, quercetin, stilbene derivatives (SITS, DIDS ($\text{IC}_{50} < 10 \mu\text{M}$), DES (maximum 30% inhibition with $\text{IC}_{50}' = 5 \mu\text{M}$)) (Fig. 2B), and activated strongly by bicarbonate and sulfite and slightly by chloride. However, it was insensitive to pyridoxal phosphate like liver enzyme.

We therefore, tried to identify ATPase(s) on polyacrylamide gel. ATPase(s) were solubilized from membranes using C_{12}E_9 as detergent, and analyzed on undenatured polyacrylamide gel electrophoresis (PAGE) by activity staining technique (Fig. 3). Two efraeptin-insensitive ATPase bands (500–650 KDa [ATPase I] and 360 KDa [ATPase II]) were recognized in liver enzymes, in contrast to apparently single main band (≈ 650 KDa) accompanied by a faint band (≈ 550 KDa) in kidney enzymes. The ATPase I band was highly sensitive to NEM and not expressed when Mg^{2+} was replaced with low concentration ($10 \mu\text{M}$) of Ca^{2+} . [^{14}C]DCCD, a specific probe for H^+ -translocating ATPase (H^+ -ATPase), migrated very close to ATPase I band, but bafilomycin-sensitive ATPase activity was hardly detected on PAGE (manuscript in preparation).

ATPase II band of rat liver lysosomes was resistant to NEM and strongly inhibited by La^{3+} and was expressed even in the absence of Mg^{2+} and with low concentration of Ca^{2+} . The function of the ATPase II is not clear, at the moment. We investigated the possible Ca^{2+} -pumping activity on rat liver lysosomes with negative results, at least in the condition where Ca^{2+} -pumping activity was detected on polymorphonuclear leucocyte lysosome fractions (9) (manuscript in preparation).

FIG. 3. Activity staining on undenatured polyacrylamide gel of ATPases of lysosomes from two different origins. Mg^{2+} -ATPase activity was stained with lead nitrate method at pH 8.5 in the presence of efraeptin ($5 \mu\text{g/ml}$).

IV. PROPERTIES OF LYSOSOMAL H⁺-ATPASE

The clarification of the structure of lysosomal H⁺-ATPase has been hampered by its highly unstable nature and that it is rather hard to dissociate it from ATPase II. Recently, we have succeeded in its purification from rat liver tritosomes by solubilization with C₁₂E₉ and glycerol density gradient centrifugation and/or preparative gel electrophoresis. It is an NEM-sensitive ATPase with broad pH-activity profile showing several putative subunit proteins on SDS-PAGE including 72, 58, 37, 33, and 16 KDa, of which 16 KDa protein is a [¹⁴C]DCCD binding protein.

Recently, the structure and function of the subunit proteins have been clarified for some of the vacuolar type H⁺-ATPases. These studies suggest that the vacuolar H⁺-ATPases (V-type ATPases) have structures with both similarities with and differences from those of F₀F₁-type (F-type) H⁺-ATPases (6). The putative subunit composition we identified on lysosomal H⁺-ATPase is in good agreement with the hypothesis that the lysosomal H⁺-ATPase belongs to V-type ATPase. We are currently investigating the reconstitution of proton pump activity using purified enzyme to see if it functions to transport protons across membranes.

V. TRANSPORT FUNCTIONS OF LYSOSOMES

Great progress has also been made in the study of other secondary transport functions on lysosomal membranes. They include transport of low molecular weight products of intralysosomal digestion, for example amino acids (basic, neutral and acidic), sugars (glucose, galactose, mannose, sialic acid, aminosugars), nucleosides (adenosine, inosine, uridine), as well as transport of anions (chloride) and cations (potassium) associated with proton movement (7). Transport systems are also sug-

TABLE 2A. *Effect of chemicals on the transport activity of rat liver tritosomes for D-glucose and L-arginine. Rat liver tritosomal membrane vesicles were incubated at 37°C for 30 sec, filtered through Millipore filter (0.22 μm) and their radioactivities remaining on the filter were counted in a liquid scintillation counter.*

Addition	conc.	Uptake of [³ H] D-Glucose (% of control)	Uptake of [³ H] L-Arginine (% of control)
None (control)		100	100
Phloretin	100 μM	67 ± 12	115 ± 16
NEM	1 mM	71 ± 12	102 ± 19
PCMB	1 mM	68 ± 5	68 ± 4
DIDS	100 μM	103 ± 0	—
SITS	100 μM	97 ± 28	—
Pyridoxal 5-phosphate	1 mM	89 ± 27	—
N-bromosuccinimide	1 mM	22 ± 8	34 ± 4
Phenylglyoxal	10 mM	65 ± 8	—
DFP	100 μM	86 ± 2	113 ± 7
Diethylpyrocarbonate	1 mM	112 ± 28	80 ± 7
NaN ₃	10 mM	57 ± 0	104 ± 3
KCN	10 mM	97 ± 2	111 ± 16

TABLE 2B. *Effect of non-radioactive substrates on the transport activity of rat liver tritosomes for L-arginine and L-glutamic acid. See Table 2A for details.*

Addition (1 mM)	Uptake of [³ H] L-Arginine (% of control)	Addition (1 mM)	Uptake of [³ H] L-Glutamic acid (% of control)
Exp. 1		Exp. 2	
None (control)	100	None (control)	100
L-Arginine	46 ± 13	L-Glutamic acid	54 ± 4
D-Arginine	78 ± 1	D-Glutamic acid	72 ± 4
L-Lysine	68 ± 0	L-Arginine	85 ± 1
L-Glutamic acid	118 ± 19	L-Leucine	99 ± 11
L-Leucine	92 ± 2	L-Proline	111 ± 1
L-Methionine	110 ± 12	Exp. 3	
L-Histidine	97 ± 12	None (control)	100
L-Proline	200 ± 49	D-Glutamic acid	84 ± 25
		L-Leucine	95 ± 9
		L-Aspartic acid	54 ± 7

gested on lysosomal membranes for cyanocobalamin (vitamine B₁₂) and cholesterol. ATP hydrolysis and consequent generation of pH- and membrane potential-difference has been shown to be associated with effective egress of some metabolites from lysosomes.

Fig. 4 and Tables 2A and 2B show selected data of our results on the transport activities of lysosomes (tritosomes) for amino acids, sugar, nucleoside and so on. They showed time-dependent, saturable kinetics with substrate specificity (except adenine transport), and inhibited by some chemical modifications, showing the presence of specific carrier molecules on lysosomal membranes. Transport properties of lysosomes for metabolites (e.g. sugars) are also confirmed by a newly established simple assay procedure using FD-fluorescence (manuscript in preparation).

VI. ATP-DEPENDENT LYSIS OF LYSOSOMES AND MEMBRANE FUSION IN CELL-FREE SYSTEM.

There is another type of important transport function associated with lysosomes, which is mediated by the membrane fusion reactions with lysosomes themselves, endosomes, autophagosomes or secretory granules.

We established a candidate cell-free system for the analysis of lysosomal membrane fusion, where lysosomes are disrupted by the combined addition of basic substances (or acidic ionophores) and ATP as a consequence of their failure to fuse with each other (submitted for publication). The disruption of lysosomes, which was detected by the release of FD from lysosomes, was a consequence of their osmotic swelling induced by protonated bases (or cations specific for particular ionophores) accumulated within lysosomes by the action of proton pump activity (3, 18). Table 3 summarizes the properties of the ATP-dependent lysis of lysosomes. This phenomenon can be regarded as an *in vitro* manifestation of vacuole formation caused by basic substances or acidic ionophores *in vivo* (13).

FIG. 4. Time course of uptake into lysosomes of L-arginine, L-glutamic acid, L-cystine, adenosine, adenine and D-glucose. Tritosomal membrane vesicles were incubated at 37°C in 0.1 M KCl, 0.2 M sucrose, 40 mM Mops-TMAH (pH 7.0) with radiolabelled substrate, collected on Millipore filter (0.22 μ m) and the radioactivities remaining on the filters were counted in a liquid scintillation counter. ○: 0°C, control; ●: 37°C, control; ■: 37°C, +cold L-amino acid or adenosine (1 mM) added at 5 min; ▲: 37°C, +cold D-amino acid (1 mM) added at 5 min.

In this system, addition of cytosol suppressed lysosome disruption (Fig. 5). This phenomenon is most reasonably explained by the requirement of cytosolic factor(s) for membrane fusion between lysosomes themselves (4, 13). The factor(s) were found to be heat-labile, NEM-resistant proteinaceous substance(s) of high-molecular weight.

Recently, *in vitro* endosome-lysosome fusion has been reported to require heat labile, NEM-resistant cytosolic factor(s) with similar properties (11), in contrast to the requirement of NEM-sensitive factor for early endosome fusion (5). Membrane-fusion is a prerequisite for the function of vacuolar system and the clarification of its regulatory mechanism should give us useful information for understanding and con-

TABLE 3. *Properties of ATP-dependent lysis of lysosomes induced by basic substances or acidic ionophores.*

1. Any of the vacuoligenic bases or acidic ionophores, but none of the non-vacuoligenic bases or neutral ionophores induces disruption of lysosomes.
2. Disruption of lysosomes requires functional proton pump and is inhibited by the inhibitors of lysosomal proton pump including DCCD.
3. Disruption of lysosomes requires permeant anion and is suppressed when permeant anion is replaced with impermeant one.
4. Disruption of lysosomes is also suppressed when the osmotic pressure of suspending medium is increased.

FIG. 5. Effect of cytosol on the ATP-dependent lysis of lysosomes by nigericin. For the details of incubation, see ref. 10.

trolling multicellular organisms including human beings. Studies of this line are currently in progress in our laboratory.

Another suggestion in the above phenomenon is the possible continuity of lysosomal compartment *in vivo*, which is disrupted in isolated fractions. In fact, the existence of tubular lysosomes has been reported recently in a variety of cell types (22). The continuity of lysosomal compartment could allow accommodation of a large amount of basic substances within the lysosomal system and its disruption, certainly, should result in osmotic lysis of lysosomes because of limited available volume. Further studies are required and in progress for full explanation of this interesting *in vitro* phenomenon.

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