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THE ACIDIFICATION OF RAT LIVER LYSOSOMES IN VITRO: A ROLE FOR THE MEMBRANOUS ATPase AS A PROTON PUMP

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SUMMARY

Lysosomes were purified from the livers of rats which had been treated with Triton WR-1339. The ATPase activity of these lysosomes was stimulated by preincubation with NaCl or KCl, conditions which diminish the proton gradient due to Donnan equilibrium. Subsequent to this preincubation measurements of methylamine uptake by lysosomes showed an ATP-dependent enhancement. Simultaneous measurements of the internal volumes of lysosomes confirmed that ATP-dependent methylamine uptake is due to acidification of lysosomes by 0.3 to 0.5 pH units. Because the conditions which stimulated ATP-dependent methylamine uptake also stimulated the ATPase activity it is concluded that acidification of lysosomes requires an ATPase which functions as a proton pump.

The internal pH of lysosomes is known to be acidic; various measurements indicate a pH of about 5 *in vivo* (1-4). Two different mechanisms for acidification of lysosomes have been proposed. On the basis of a preferential permeability of rat liver lysosomal membranes to protons measured *in vitro*, Tager and coworkers proposed that an internal pH, one unit below that of the medium, exists by virtue of Donnan equilibrium (5,6). However, Mego and coworkers observed that proteolysis in intact lysosomes *in vitro* was stimulated by addition of ATP and, accordingly, proposed the existence of an ATP-driven proton pump (7). We have previously examined the lysosomal membrane and shown that it contains a relatively high ATPase activity,

ABBREVIATIONS

ATPase, Adenosine triphosphate phosphohydrolase, EC No. 3.6.1.3; MOPS, morpholinopropane sulfonate; EDTA, (ethylenedinitrilo) tetraacetic acid; EGTA, ethyleneglycol-bis (β-aminoethyl ether)-N,N'-tetraacetic acid; C-CCP, carbonyl cyanide, m-chlorophenylhydrazone; AMP-PNP, adenylyl imidodiphosphate.

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1 μmol/min-mg of protein (8,9,10). Results indicative of an ATP-driven proton pump in rat liver lysosomes are presented here.

METHODS

Lysosomes were isolated from livers of rats which had been treated with Triton WR-1339 as described (10).

ATPase activity was measured by incubating the sample in 2 mM $MgCl_2$, 1 mM ATP, 0.5 mg of bovine serum albumin per ml, salt as indicated, and 20 mM morpholinopropane sulfonate, pH 7.0, at 37° for 10 min. The assay was terminated by addition of trichloroacetic acid. The samples were clarified by centrifugation, and phosphate was determined by adding ascorbate-ammonium molybdate to an aliquot of each supernate (11).

Methylamine uptake was measured after incubation of [^{14}C]-methylamine with intact lysosomes at room temperature in various media as described in the table legends. After incubation, the lysosomes were pelleted by centrifugation in a Brinkmann Model 3200 Microfuge for 2 min, and the radioactivity of each supernate and pellet was determined. Parallel incubations of [^{14}C]-sucrose and [^{3}H]-water with intact lysosomes at room temperature in each of the described media were performed simultaneously. Internal volumes of each sample were determined by subtracting the sucrose space from the water space as described by Tager and coworkers (5,6). Supernates were mixed directly with Triton-toluene and analyzed in a Packard scintillation counter. Pellets were dissolved in 500 μl of 0.2 N NaOH; the solution was then acidified by addition of 75 μl of 40% trichloroactic acid and clarified by centrifugation. An aliquot of each trichloroacetic acid supernate was counted in Triton-toluene; each pellet was analyzed for protein in the Lowry assay.

RESULTS

Intact lysosomes exhibit ATPase activity that is stimulated 2-fold by preincubation in either KCl or NaCl (Table I). These results could be explained if the ATPase was latent and if salts were ineffective in providing osmotic protection. If the ATPase were merely latent then ultrasonic disruption of lysosomes in sucrose should be as effective as in salt. The results in Table I show that greater activity is achieved when salts are present during disruption. Furthermore, gramicidin D and uncoupler stimulated ATPase activity (Table II) under conditions in which the latency is unaffected as determined by earlier measurements of N-acetyl-glucosaminidase (9). Thus, it is reasonable to reject the possibility that the active site of the ATPase for ATP is only exposed on the internal surface of the lysosomal membrane, and the explanation for the effect of salts appears to be related to trans-membrane gradients.

Table I. Effect of Salt and Sonication on ATPase Activity of Lysosomes

Preincubation	ATPase Activity (μmol/min-mg)	
	(-)	(+) Sonication
Sucrose	0.13	0.28
NaCl	0.35	0.41
KCl	0.38	0.43

Lysosomes were preincubated with 20 mM MOPS, pH 7.0, 5 mM EGTA and 150 mM NaCl, KCl or sucrose for 20 min. Where indicated sonication was for 2 min in a Branson model B-12 bath sonifier. Lysosomes were then diluted 10-fold to give 250 mM sucrose, 2 mM $MgCl_2$, 20 mM MOPS, pH 7.0, 1 mM ATP, 0.5 mg/ml bovine serum albumin, 0.5 mM EGTA and 15 mM NaCl or KCl and assayed for ATPase activity.

Table II. Effect of Gramicidin and Uncoupler on ATPase Activity of Lysosomes

Salt	Addition	ATPase Activity (μmol/min-mg)	
		(-)	(+) C-CCP
NaCl	-	0.25	0.36
KCl	-	0.36	0.38
KCl	gramicidin	0.42	0.47

Lysosomes were assayed for ATPase activity by incubation in 20 mM MOPS, pH 7.0, 0.5 mM EGTA, 0.5 mg/ml bovine serum albumin, 1 mM MgATP, 100 mM NaCl or KCl, 10 μg/ml gramicidin D and 2 μM C-CCP uncoupler as indicated. The incubations were terminated and products analyzed as in Table I.

The existence of a proton pump was tested by measuring the uptake of the permeant weak base, [^{14}C]-methylamine. Methylamine uptake by lysosomes has been used previously to measure intralysosomal pH (5,6). If penetration of salt diminished a Donnan-equilibrium proton gradient, then one might expect that preincubation of lysosomes in salt would lower methylamine uptake. The results obtained by Tager and coworkers support this expectation (5,6). If the ATPase is a proton pump, then lysosomes preincubated in salt should take up more methylamine in the presence of ATP. The results presented in Table III show that ATP does facilitate methylamine uptake by more than 2-fold. It is necessary to point out that the calculation of the

Table III. Methylamine Uptake by Lysosomes

Addition	Methylamine Uptake (cpm/mg protein / (cpm/µl original mixture))	((cpm/µl internal volume) / (cpm/µl original mixture))	Internal Volume (µl/mg protein)	Calculated pH_{in}
None	221.7	25.3	8.76	5.60
$+Mg_2ATP$	253.9	36.5	6.96	5.44
KCl	38.7	7.06	5.48	6.15
$+Mg_2ATP$	110.6	25.4	4.35	5.60
LiCl	34.8	17.5	1.99	5.76
$+Mg_2ATP$	86.2	59.4	1.45	5.23

Lysosomes were preincubated in 20 mM MOPS, pH 7, 0.2 M sucrose, 0.1 M salt, 7 µM [^{14}C]-methylamine (55.5 mCi/mmol) and 0.4 mM EDTA for 20 min at room temperature. Then MgATP was added to give 2 mM $MgCl_2$ and 1 mM ATP. After an additional 10 min incubation, lysosomes were collected by centrifugation for 2 min in a Brinkmann Model 3200 microfuge. Simultaneous, parallel incubations with [^{14}C]-sucrose were carried out as described in Methods and were necessary to determine the internal volume.

internal pH of lysosomes is greatly affected by changes in internal volume. After preincubation of lysosomes in salt, the internal volumes differed depending on the salt used, the more permeant the salt the greater the internal volume. The addition of ATP did not cause an increase in internal volume. The internal volume values shown are representative and conservative; in general the internal volume was 20% smaller after addition of ATP. Since lysosomes behave as osmometers (12), these results imply that ATP does not facilitate extensive solute efflux from lysosomes. Mg_2EDTA, Mg_2AMP-PNP and Ca_2ATP were not effective in promoting methylamine uptake; furthermore, results obtained with K_2SO_4 and NaCl were similar to those with KCl and LiCl. The lack of cation specificity suggests an electrogenic proton pump.

The conditions employed to demonstrate ATP-dependent methylamine uptake by lysosomes (Table III) consist of preincubation with salt and EDTA. Since EDTA in excess of added $MgCl_2$ is known to be an effective inhibitor of the ATPase (8), it is necessary to show that the ATPase is indeed active in the conditions employed before concluding that it is responsible for the enhanced uptake. The results of Table IV show that the ATPase is active in the presence of 0.5 mM EGTA and 1 mM $MgCl_2$ or in the presence of 0.5 mM EDTA and 2 mM $MgCl_2$, the conditions employed to demonstrate ATP-dependent methylamine uptake. Therefore, it is concluded that ATP-dependent uptake of methylamine by lysosomes occurs because the ATPase acts as a proton pump.

DISCUSSION

The uptake of methylamine by isolated rat liver lysosomes preincubated in salt was stimulated by addition of MgATP. Since the distribution of methylamine is directly proportional to the proton gradient (5,13), it is reasonable to conclude that the stimulated uptake represents acidification of the lysosomal interior by the membranous ATPase acting as a proton pump. It is significant to note that this evidence for ATP-dependent acidification contrasts with that obtained by Tager and coworkers (5,6). They conclude that a Donnan equilibrium alone is sufficient to explain intralysosomal pH.

Table IV. ATPase Activity of Lysosomes in the Presence of EGTA and EDTA

	ATPase Activity (μmol/min-mg)
1 mM $MgCl_2$	0.51
plus 0.5 mM EGTA	0.50
2 mM $MgCl_2$	0.54
plus 0.5 mM EDTA	0.58

Frozen lysosomes were thawed and assayed for ATPase as in Table I except that EGTA, EDTA and $MgCl_2$ were varied as indicated below and ATP was 1 mM.

Explanations for the difference in results are: (1) The ATP-enhanced uptake reported here requires a preincubation of the lysosomes in salt, a treatment which diminishes the Donnan equilibrium proton gradient; Tager and coworkers did not preincubate in salt (6). (2) Their prolonged incubations were done with 10 mM ATP (6), a condition which results in inactivation of the ATPase (8). (3) They report that ATP increases the internal volume whereas the findings reported here are that ATP has little effect on internal volume measured 10 min after addition of ATP (Table III). Internal volume measurements may be a major source of error in both laboratories; an intrinsic deficiency of the measurements is the fact that they are not done on the same sample as the methylamine uptake measurements but on samples run in parallel. The use of probes not dependent on internal volume measurements would be desirable.

The observation that addition of ATP to lysosomes results in little shrinking of the internal volume indicates that solute is not being pumped out of lysosomes. Therefore, if the ATPase is a proton pump, it probably operates without pumping monovalent cations in the outward direction.

It is necessary to point out that the results presented here do show the existence of a pH gradient in lysosomes prior to the addition of ATP. The Donnan effect may help to maintain the gradient generated by the proton pump.

At least this seems to be the case *in vitro*, whether the same is true in the cell environment remains to be proven.

The existence of an ATP-driven proton pump in lysosomes could explain in part the known energy requirement of protein degradation (14). The pump would also allow for the generation of a proton gradient in the cytoplasmic environment despite the presence of nearly 100 mM potassium ion that would tend to collapse a proton gradient created by Donnan equilibrium. The concept of a pump is consistent with the energy-dependent accumulation of weak bases in lysosomes *in vivo* (3,4), with the time dependent acidification of phagolysosomes in leukocytes (2), and with recently published observations on ATP dependent dye uptake by a mitochondrial-lysosomal fraction (15).

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