Biochimica et Biophysica Acta 899 (1987) 276–284
Elsevier

BBA 73544

The lysosomal H^+ pump: 8-azido-ATP inhibition and the role of chloride in H^+ transport

John Cuppoletti [a], Dorothea Aures-Fischer [b] and George Sachs [b,c]

[a] *Department of Physiology and Biophysics, University of Cincinnati, College of Medicine, and Veterans Administration Medical Center, Cincinnati, OH,* [b] *CURE Research Services, Veterans Administration West Los Angeles Hospital Center, Wadsworth Division, and* [c] *Department of Medicine, University of California, Los Angeles, CA (U.S.A.)*

(Received 5 January 1987)

Key words: Proton pump, electrogenic; Proton transport; ATPase, Mg^{2+}-stimulated; 8-Azido-ATP inhibition; Chloride effect; (Lysosome)

Lysosomes (tritosomes) were purified from the livers of rats injected with Triton WR 1339. The lysosomes developed an Mg^{2+}-ATP-dependent pH gradient as measured by Acridine orange accumulation. H^+ transport was supported by chloride, but not sulfate, and was independent of the cation used. H^+ transport and Mg^{2+}-stimulated ATPase was inhibited by diethylstilbesterol ($K_{0.5} = 2$ μM). *N*-Ethylmaleimide inhibited H^+ transport ($K_{0.5} = 30$ μM). At low concentrations of *N*-ethylmaleimide, ATP partially protected H^+ transport from inhibition with *N*-ethylmaleimide. Photolysis with 8-azido-ATP inhibited H^+ transport and Mg^{2+}-stimulated ATPase activity. Under these same conditions, 8-azido-[α-^{32}P]ATP reacted with a number of polypeptides of the intact lysosome and lysosomal membranes. Pump-dependent potentials were measured using the fluorescent potential-sensitive dye, $DiSC_3(5)$ (3,3′-dipropylthiocarbocyanine) and ATP-dependent potential generation was inhibited by diethylstilbesterol. Chloride, but not sulfate reduced the magnitude of the ATP-dependent membrane potential, as measured using merocyanine 540. The chloride conductance, independent of ATP, was of sufficient magnitude to generate a H^+ gradient driven by external chloride in the presence of tetrachlorosalicylanilide. In Cl^- free media, ATP-dependent H^+ transport was restored to control levels by outwardly directed K^+ gradients in the presence of valinomycin. The role of cell Cl^- is to provide the necessary conductance for supporting lysosomal acidification by the electrogenic proton pump.

Introduction

Several classes of organelles have been demonstrated to maintain pH gradients. Amongst these are the chromaffin granules [1,2], Golgi membranes [3,4] and clathrin-coated vesicles [5,6]. The lysosomal enzymes have a pH optimum in the region of pH 5, suggesting that the lysosomes are acidified [7]. Lysosomal ATPase was first deduced from the finding that protein degradative function requires an ATPase activity with an alkaline pH optimum [8]. A membrane-associated alkaline ATPase activity was described by Schneider [9]. Pump function was studied in intact, living cells [10], and in intact or disrupted lysosomes [11–14]. The lysosomal membrane proteins including the ATPase have been solubilized [15]. Antibodies

Abbreviations: $DiSC_3(5)$, 3,3′-dipropylthiocarbocyanine; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; Pipes, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride.

Correspondence: J. Cuppoletti, Department of Physiology and Biophysics, University of Cincinnati, College of Medicine, Cincinnati, OH 45267–0576, U.S.A.

have been prepared against lysosomal membranes [16].

The H^+ pump isolated from the various organelles appear to be quite similar in that pH differences of 2–3 pH units are generated, they are insensitive to ouabain and vanadate, and are relatively insensitive to inhibitors of the mitochondrial pump. The organellar H^+ transport pumps, including the lysosomal pump are relatively insensitive to 10^{-4} M DCCD (*N,N'*-dicyclohexylcarbodiimide) [17,18], and all are sensitive to *N*-ethylmaleimide to some extent [12,19,20], suggesting similarities amongst the various organellar H^+ pumps. On the other hand, some differences in pump responses to inhibitors, energy sources and in the nature of the anion requirement have been noted. These may reflect differences in the pumps, a possibility which may only be confirmed by comparing the various pumps in purified form [6,21]. Duramycin is a potent inhibitor of the clathrin-coated vesicle pump [22] yet stimulates a vanadate- and ouabain-insensitive pump from neutrophils [17]. Quercetin and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole do not inhibit the lysosomal alkaline ATPase [12] but do inhibit the clathrin-coated vesicle pump [23] and quercetin inhibits neutrophil granule acidification [17]. ITP and GTP are not substrates for the clathrin-coated vesicle pump [21], but appear to be substrates for the lysosomal ATPase [12], although nucleoside diphosphokinase activities may be present in lysosomal preparations [21]. Halides appear to support H^+ pump activity by serving as a counterion in clathrin-coated vesicles from bovine brain [21], and in Golgi membranes [4]. On the other hand, with rat liver clathrin-coated vesicles, significant pump activity occurs in the absence of added salts other than substrate levels of disodium ATP and $MgSO_4$ [25]. In the lysosome, both phosphate [26] and chloride [12] have been suggested as possible counterions.

Very recent successes in the isolation, purification, and characterization of the H^+ pumps from the clathrin-coated vesicle have been achieved relying upon inhibitor sensitivity of the solubilized and reconstituted pump activity to determine the degree of purification [27]. Similar dramatic results have been obtained for the pump from the chromaffin granule [28] using similar methods. As will be discussed, prior to attempts at purification and identification of the lysosomal proton pump, it was desirable to initiate a search for potential covalent tags of the lysosomal pump, and to further examine the nature of the anion permeability pathway in the lysosome. The latter would be particularly important to future studies of the lysosomal proton pump should the ATPase activity be separable from the putative anion permeability pathway upon treatment with detergents or inhibition of the chloride pathway [21].

Accordingly, in this paper, diethylstilbesterol is shown to be a potent inhibitor of the lysosomal proton pump and photolysis with 8-azido-ATP is inhibitory to the pump, providing a potential covalent tag of the ATP binding subunit of the pump. Further, under the conditions of our experiments, a major determinant of the acidification of lysosomes and lysosomal membranes is an anion conductance (which can carry chloride), necessary for the ATP-dependent accumulation of acid by an electrogenic H^+ pump [29]. In the absence of chloride, outwardly directed potassium gradients with valinomycin provide adequate conductance.

Materials and Methods

Materials. Radioactive 8-azido-[α-^{32}P]ATP and [γ-^{32}P]ATP were obtained from ICN, Inc. Valinomycin, nigericin, *p*-Iodonitrotetrazolium violet, *p*-nitrocatechol sulfate, Triton WR 1339 (Tyloxapol) and azido-ATP were obtained from Sigma. $DiSC_3(5)$ (3,3'-dipropylthiocarbocyanine iodide) was obtained from Molecular Probes, Junction City OR, merocyanine 540 was obtained from Eastman. All other reagents were of the highest grades available commercially.

Enzyme preparation. Rat liver lysosomes were prepared from Triton WR 1339-injected rats [30] as modified by Trouet [31]. Lysosomes, isolated from sucrose gradients, were quick-frozen as suspensions in solid CO_2-methanol mixtures, stored at $-80\,^\circ$C, and the samples were rapidly thawed in flowing water. 1 mM EDTA was included for the preparation of lysosomes. Freeze-thawed membranes were prepared by resuspending and washing quick-frozen lysosomes in 300 mM sucrose medium at $0\,^\circ$C containing 8 mM Pipes, 4

mM Tris (pH 7.4), and collected by a 100 000 × *g* centrifugation for 20 min. 1 mM EDTA and 1 mM PMSF (phenylmethylsulfonyl fluoride) were routinely included in all lysis media. In some instances α-tosyl-lysyl chloromethane was included. Osmotically lysed membranes were prepared by resuspending quick-frozen lysosomes in 20 volumes of 4 mM Pipes, 8 mM Tris (pH 7.4) and centrifuging at 100 000 × *g* for 20 min.

Determination of marker enzymes. Acid phosphatase was measured by following the rate of hydrolysis of *p*-nitrophenylphosphate [31] and 2-glycerol phosphate at pH 5.0 [32]. Aryl sulfatase A + B was measured by following the rate of hydrolysis of nitrochatechol sulfate [33]. Succinic dehydrogenase activity was estimated by following the rate of reduction of *p*-Iodonitrotetrazolium violet [34] and by following phenazine methosulfate reductase activity [35]. In four representative experiments, the relative specific activity of aryl sulfatase A + B was 27.2 ± 1.2, aryl sulfatase A was 25.7 ± 1.1, 2-glycerol phosphatase was 31.1 ± 2.7, and acid phosphatase was 23.0 ± 0.08.

Mg^{2+}-ATPase activity. Mg^{2+}ATPase activity was determined using the method of Yoda and Hokin [36] or a more sensitive modified procedure employing [γ-^{32}P]ATP. Briefly, 1–20 μg of protein was added to 1 ml reaction mixture containing (final): 4 mM Pipes, 8 mM Tris (pH 7.4), 1 mM ATP or [γ-^{32}P]ATP (10^5 dpm/μmol), containing 4 mM $MgCl_2$ and adjusted to pH 7.4 with Tris base. Inhibitors were added as indicated. Blanks containing in addition 10 mM EDTA pretitrated to pH 7.4 with Tris base were used to determine total Mg^{2+}-stimulated ATP hydrolysis. Reactions were carried out at 37 °C for 30 min – 1 h, and terminated by the addition of 1 volume of 0 °C stop solution containing 3.6% ammonium molybdate, 15% perchloric acid. The colored (or radioactive) phosphomolybdate complex was extracted into 2.5 volumes of *n*-butylacetate at 0 °C. The extent of ATP hydrolysis was then determined from the absorbance at 320 nm or by the radioactivity of the organic phase (1 ml) counted in 10 ml Liquiscint scintillation fluid.

Proton transport. H^+ transport activity was determined in intact lysosomes (50–100 μg), or in freeze-thawed membranes by following the rate and extent of change of absorbance of Acridine orange (ΔA 546–492 nm) in an Aminco DW-2 spectrophotometer [37]. H^+ transport reactions were carried out at 22 °C in 1 ml of reaction mixture containing 0.3 M sucrose, 50–100 mM KCl or tetramethylammonium Cl^-, 4 mM Pipes, 8 mM Tris (pH 7.4) and 10 μM Acridine orange. Reactions were initiated by the addition of 1 mM ATP-Mg prepared by titration to pH 7.4 with Tris base. To measure rates of H^+ leak, lysosomes were allowed to accumulate H^+ under standard conditions to steady-state levels of H^+, and the transport reaction was abruptly terminated by chelating Mg^{2+} with buffered EDTA (10 mM). The rate of loss of accumulated acid was determined from the characteristic $t_{1/2}$ for return to the initial starting Acridine orange absorbance. Semiquantitative information regarding effectors of H^+ leak rate could then be obtained by comparing the observed $t_{1/2}$ for H^+ leak with that obtained for the accumulation of H^+ in the same set of experiments. In most experiments, nigericin (0.5 μg/ml) or tetrachlorosalicylanilide (0.3 μM) plus valinomycin (1 μg/ml) were used to dissipate accumulated H^+.

Membrane potential. The change in fluorescence of $DiSC_3(5)$ (excitation at 620 nm, emission at 675 nm) was used to determine the potential which developed in the intact lysosomes following ATP-Mg^{2+} addition. Lysosomes were incubated in 1 ml reaction mixtures containing 0.3 M sucrose, 4 mM Pipes, 8 mM Tris (pH 7.4), and 1 μM $DiSC_3(5)$ [13]. The fluorescence of the dye was continuously monitored on an MPF-44 spectrofluorimeter at 22 °C. The negatively charged potential sensitive dye, merocyanine 540, (excitation at 540 nm, emission at 570 nm) was used to estimate the relative membrane permeabilities for anions, with and without ATP-Mg. The enhancement or quench of fluorescence of 5 μM merocyanine 540 solutions bathing lysosomes was monitored following additions of ATP-Mg^{2+}, tetramethylammonium Cl^-, or tetramethylammonium $SO_4^=$ to the reaction mixture.

Azido-ATP. Inhibition of Mg^{2+}-stimulated ATPase activity with azido-ATP and azido-[α-^{32}P]ATP affinity labeling [38] of lysosomes and membranes derived from lysosomes were carried out in quartz cuvettes using a 150 W Xenon lamp for photolysis. Reaction mixtures contained 6 mM

$MgCl_2$, 4 mM Pipes, 8 mM Tris (pH 7.4) at 0 °C. 0.5 mM PMSF was included. Substrates for photolysis were (0.05 μCi/ml) (4 μM) azido-[α-^{32}P]-ATP (with or without 250 μM ATP), or 250 μM ATP alone. Irradiations were carried out under standard conditions (usually 1 min) that did not lead to inactivation of the Mg^{2+}-stimulated ATPase, nor inhibition of H^+ accumulation by light alone (see below). For estimation of the amount of label incorporated into specific polypeptides, samples were treated following irradiation with 1 volume of ice-cold 10% trichloroacetic acid containing 250 μM ATP, and the samples were centrifuged in Eppendorf centrifuge tubes at 12000 rpm for 3 min. Samples were then washed twice with 1 ml ice-cold 5% trichloroacetic acid. Lipids and remaining trichloroacetic acid were removed with a single wash in chloroform/methanol (1 : 1, v/v) or ethanol/dipropyl ether (1 : 1) prior to analysis by sodium dodecyl sulfate gel electrophoresis and autoradiography of the resultant electrophoretograms.

Miscellaneous. Protein was determined by the method of Lowry et al. [39]. Sodium dodecyl sulfate gel electrophoresis was carried out by the method of Laemmli [40] on 10% acrylamide slab gels. Gels were stained, dried and overlaid with polyethylene film prior to autoradiography. Autoradiograms of ^{32}P-labeled protein labeled with 8-azido-[α-^{32}P]ATP were obtained with Cronex 4 film (Dupont) using High-Plus intensifying screens (Dupont). Autoradiograms were developed after 3–7 days exposure at −80 °C using a Kodak automatic X-ray film processor.

Results

Lysosome preparation

All solutions for preparation contained 1 mM EDTA. Lysosomes were intact as isolated by floatation. When lysosomes were frozen or lysed, 1 mM EDTA and 1 mM PMSF were added. The H^+ transport function was stable for several hours at 0 °C.

Proton transport

Fig. 1 shows that the lysosomes exhibited ATP-dependent H^+ transport in tetramethylammonium chloride media. Similar rates and extents

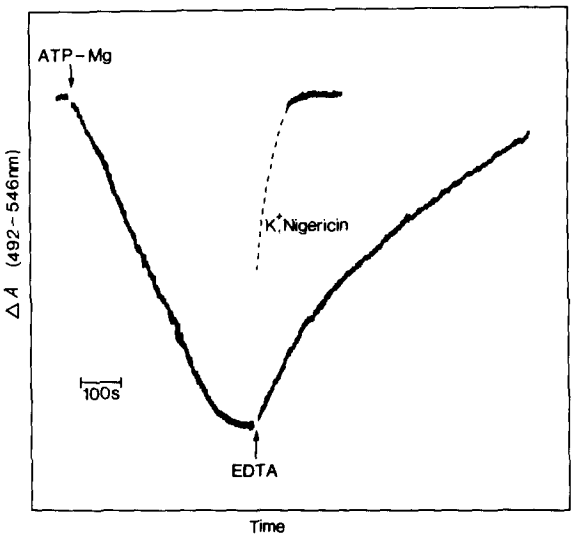

Fig. 1. Proton transport. 50 μg of frozen-thawed lysosomes were assayed at 20 °C in H^+ transport media containing 50 mM tetramethylammonium Cl^-. H^+ accumulation rates were determined from the $t_{1/2}$ for reaching steady-state H^+ accumulation. H^+ leak rates were determined similarly following addition of Tris-buffered EDTA (pH 7.4). Additions of 50 mM K_2SO_4 and 5 μg/ml nigericin were used in a separate experiment to show that Acridine orange responses were not rate-limiting.

of transport were observed with NaCl or KCl, but no transport was observed with substitution of Cl^- with sulfate or gluconate. Nigericin, an electroneutral H^+/K^+-exchange ionophore, dissipated the accumulated H^+ gradient when added together with K^+.

H^+ transport was sensitive to inhibition with diethylstilbesterol (Fig. 2A) ($K_{0.5app} = 2$ μM). At concentrations less than 10 μM, diethylstilbesterol inhibited H^+ transport without increasing H^+ leak rates. (see Materials and Methods).

N-Ethylmaleimide inhibited H^+ transport (Fig. 2B) ($K_{0.5\ app} = 30$ μM). Inhibition was partially prevented by prior addition of 1 mM α,β-methylene-ATP. Prior addition of excess β-mercaptoethanol (10 mM) or dithiothreitol (1 mM) prevented inhibition by *N*-ethylmaleimide, but would not reverse inhibition when added after *N*-ethylmaleimide (not shown).

Photolysis with 250 μM 8-azido-ATP inhibited H^+ transport (Fig. 3). Irradiation of the lysosomes without 8-azido-ATP or treatment with 8-azido-ATP in the dark did not inhibit transport.

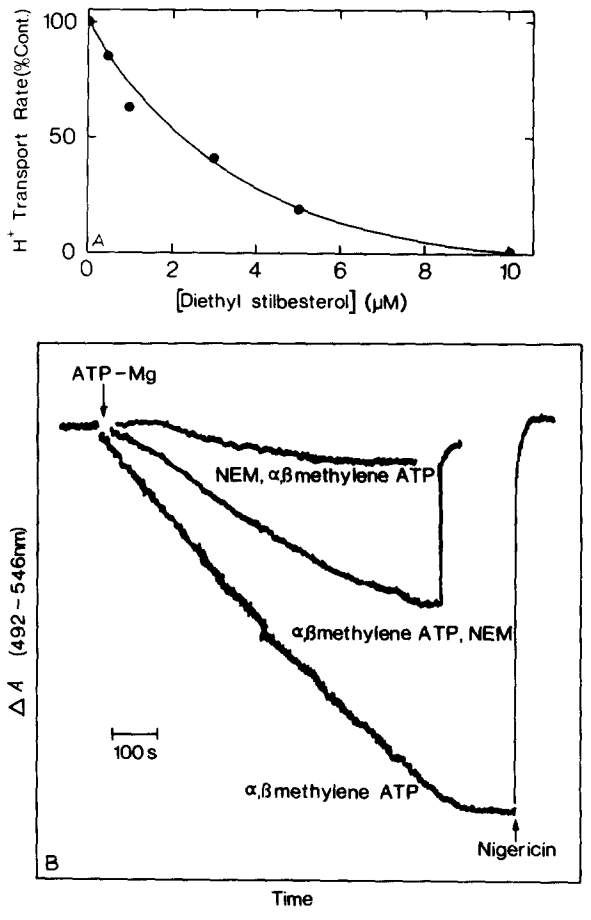

Fig. 2. Inhibitors of H^+ transport. In A, 50 μg of lysosomes were assayed for H^+ transport in media containing the indicated concentrations of diethylstilbesterol. Initial rates of H^+ transport were plotted as a percentage of the control. H^+ leak rates were not increased at these concentrations of diethylstilbesterol. In B, 50 μg of lysosomes were pretreated for 5 min at 0 °C with or without 30 μM *N*-ethylmaleimide (NEM) before or after 1 mM α,β-methylene-ATP, as indicated. Following incubation, the lysosomes were diluted and assayed for H^+ transport at 20 °C. Similar protection was afforded by 1 mM ADP-Mg^{2+} or ATP-Mg^{2+}. In control experiments not shown, inhibition was prevented by prior addition of 1 mM dithiothreitol or 10 mM β-mercaptoethanol.

Vanadate (0.10 mM), ouabain (0.5 mM), oligomycin (1 μM) and DCCD (2 μM), were not inhibitory. At higher concentrations, oligomycin (3 μM) and DCCD (50 μM) were inhibitory.

Mg^{2+}-stimulated ATPase

Isolated lysosomes and membranes prepared by hypotonic lysis exhibited Mg^{2+}-stimulated ATPase

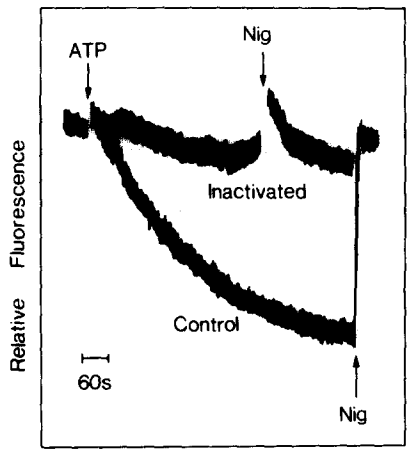

Fig. 3. Inhibition of H^+ transport with 8-azido-ATP. In the upper curve, lysosomes were assayed for H^+ transport with 250 μM 8-azido-ATP with 30 s irradiation. In the lower curve is a control experiment in which the lysosomes were first reacted in the light for 30 s, and then treated in the dark with 8-azido-ATP prior to the H^+ transport assay, showing that membrane barrier properties and transport properties were intact following light treatment and 8-azido-ATP treatment.

activity. Vanadate, (0.1 mM), ouabain (0.5 mM), oligomycin (1 μM) and DCCD (2 μM) were not inhibitory to the Mg^{2+}-ATPase. *N*-Ethylmaleimide and diethylstilbesterol only partially inhibited the Mg^{2+}-stimulated ATPase, in contrast to the complete inhibition of H^+ transport seen under similar conditions.

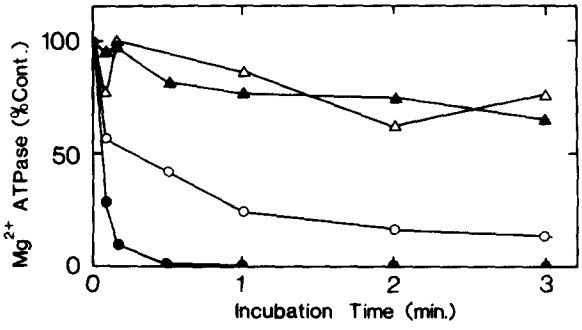

Fig. 4. Inactivation of Mg^{2+}-stimulated ATPase with 8-azido-ATP. Membranes were either incubated at 0 °C in the dark for the indicated times in the presence of 250 μM 8-azido-ATP (▲), or irradiated as described in Materials and Methods with 250 μM 8-azido-ATP (●), with 250 μM 8-azido-ATP plus 2.5 mM ATP (○) or without additions (△). Following irradiation or incubation, the membranes were diluted 100-fold for assay using the radioactive ATPase assay as described.

Photolysis with 250 μM 8-azido-ATP also inhibited Mg^{2+}-ATPase activity (Fig. 4). Inhibition in the presence of 8-azido-ATP was time and light dependent. Excess ATP slowed the time course of inactivation. Light alone, or treatment with 8-azido-ATP in the dark did not inhibit Mg^{2+}-ATPase activity. The Mg^{2+}-insensitive ATPase activity was not inhibited by photolysis with 8-azido-ATP under these conditions. Preliminary attempts to label the lysosomal membrane proteins with 8-azido-[α-^{32}P]ATP were carried out (see Materials and Methods). In lysosomes, a number of polypeptides including polypeptides of approx. 80 and 40 kDa were labeled by 8-azido-ATP, in an ATP-protected manner. Specific identification of the polpeptide which represents the ATP binding subunit(s) of the lysosomal proton pump will require further investigation.

Pump-dependent membrane potentials

The lysosomal H^+ pump is electrogenic and the potential generation responds to inhibitors [12,13]. $DiSC_3(5)$ [12,13,41], a positively charged, potential sensitive fluorescent probe of membrane potential was used to examine the sensitivity of pump-dependent potential generation to diethyl stilbesterol (Fig. 5A). Here, lysosomes were incubated with the potential probe in sucrose media. A time-dependent fluorescence quenching was observed with control and inhibitor-treated lysosomes. Upon addition of ATP-Mg^{2+}, there was a rapid enhancement of fluorescence with control lysosomes, presumably due to movement of the dye from the membrane (quenched), to free solution (fluorescent), because of the generation of an interior more positive membrane potential. Diethylstilbesterol prevented the ATP-induced potential (Fig. 5A), as did prior treatment with 100 μM *N*-ethylmaleimide [13] and photolysis with 250 μM 8-azido-ATP (not shown). None of these agents prevented the initial fluorescence quench, suggesting that the effect was on the pump.

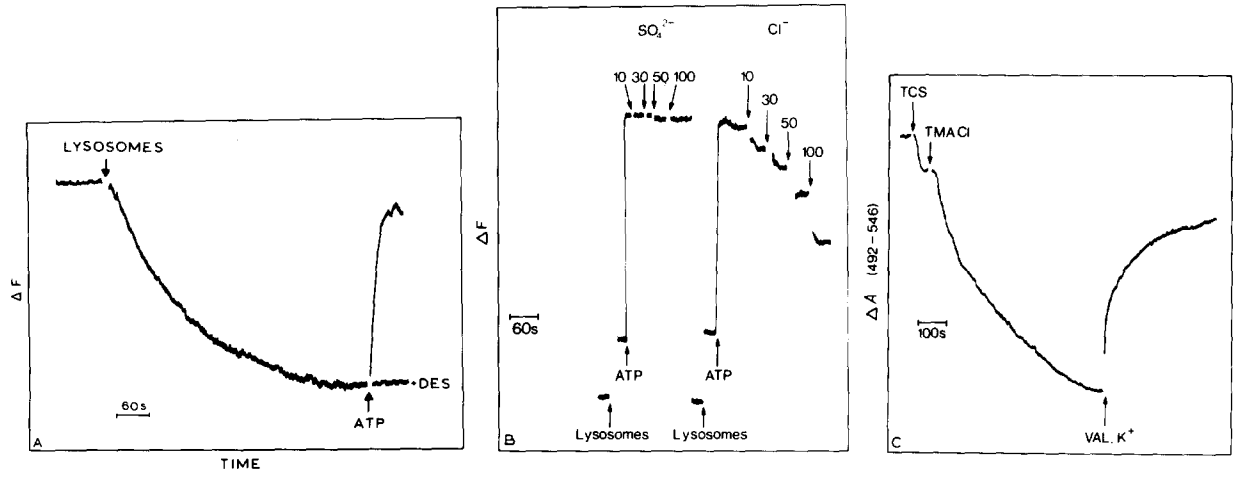

Fig. 5. Pump electrogenicity and conductive pathways. In A, 20 μg lysosomes were added to media containing 1 μM $DiSC_3(5)$ (see Materials and Methods). Uptake of the dye was allowed to reach steady state, and 1 mM ATP-Mg was added. The fluorescence response following ATP addition was prevented by 10 μM diethylstilbestereol (DES). In B, 20 μg of lysosomes were incubated at 20 °C in sucrose media containing 5 μM merocyanine 540 (see Materials and Methods). Upon addition of 1 mM ATP-Mg, a rapid fluorescence enhancement was observed. Upon subsequent addition of tetramethylammonium Cl^-, but not SO_4^{2-}, a dose-dependent fluorescence quench was observed. Similar fluorescence quench occurred in the absence of ATP. In C, 100 μg of lysosomes were incubated at 22 °C in buffered sucrose media containing 10 μM Acridine orange. 0.1 μM tetrachlorosalicylanilide (TCS) was then added, followed by 100 mM tetramethylammonium Cl^- (TMA Cl), as indicated. The rapid uptake of Acridine orange via a tetrachlorsalicylanilide induced H^+ conductance and an endogenous Cl^- conductance was dissipated by 5 μg/ml valinomycin and 50 mM K_2SO_4. For comparison, the K^+ conductance of the lysosomal membrane using 100 μg of K^+-preloaded lysosomes (prepared as described in Fig. 2) was similarly studied. With a 20-fold K^+_{in}/K^+_{out} gradient, no H^+ accumulation could be observed upon addition of TCS, but valinomycin plus TCS gave acidification (not shown). This indicates that P_K is less than P_{Cl}.

Anion conductance measurements

For a simple uniport pump, anion movement through a conductance in the membrane is required for accumulation of acid [29]. In Fig. 5B, we used the fluorescent, potential-sensitive dye, merocyanine 540, to demonstrate the existence of such a conductance in the lysosomal membrane, and to examine the specificity of the anion conductance. Upon incubation of the lysosomes with the dye, a fluorescence enhancement was observed. ATP-Mg^{2+} caused a fluorescence quench, consistent with the generation of an interior-positive membrane potential. Cl^-, but not sulfate, reduced the ATP-dependent potential in a concentration-dependent manner towards the resting potential, as previously shown for ATP generated $\Delta\psi$ using $DiSC_3(5)$ [12,13].

Fig. 5C shows an experiment investigating whether the observed Cl^- conductance was of sufficient magnitude to satisfy the requirement for net HCl accumulation during pump activity. Lysosomes were incubated briefly in sucrose media containing Acridine orange. Tetrachlorosalicylanilide was then added. Upon imposition of a large, inwardly directed Cl^- gradient, acidification was observed. The acidification was somewhat smaller, (but of similar magnitude) to that obtained during ATP-dependent proton pumping. Dissipation of the H^+ gradient was achieved with K^+ and valinomycin, which functions with the initially added tetrachlorosalicylanilide to promote H^+/K^+ exchange. In similar experiments, K_2SO_4-loaded lysosomes did not produce appreciable H^+ gradients with tetrachlorosalicylanilide, showing that K^+ conductances are finite but limited in the lysosomal membrane [13].

Role of chloride in pump function

Since proton transport was Cl^- dependent (Fig. 1), it was important to determine whether Cl^- was required for proton pump function other than for relief of ATP generated membrane potential. In Fig. 6, Cl^--independent proton transport function was measured under K^+ gradient conditions in the presence and absence of the electrogenic ionophore, valinomycin. When K_2SO_4-loaded lysosomes (see Materials and Methods) were added to media containing equimolar K_2SO_4 (no K^+ gradient), little transport was observed in the presence

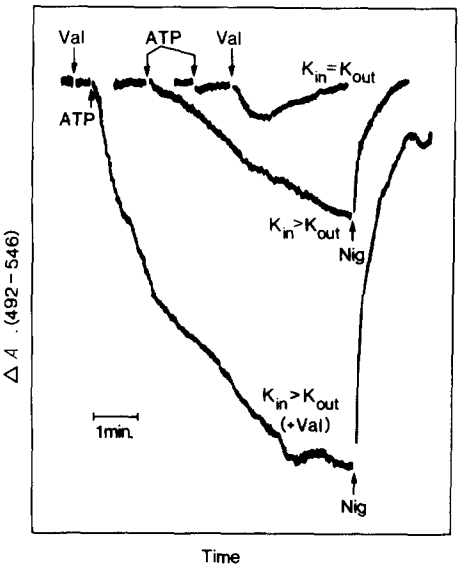

Fig. 6. Cl^--independent proton transport. 100 μg lysosomes were preloaded with K^+ by two cycles of rapid freeze-thaw and extrusion through a fine gauge needle in media containing 50 mM K_2SO_4. ATP-Mg (1 mM), valinomycin (Val, 5 μg/ml), and nigericin (Nig, 5 μg/ml) were added as indicated. The lysosomes were assayed for Cl^- independent H^+ transport by dilution into media containing different K_2SO_4 concentrations. (i) $K_{in} = K_{out}$, 50 mM K_2SO_4, (ii) ($K_{in} > K_{out}$), 0 added K^+, giving a final 20 fold in/out K^+ gradient, (iii) ($K_{in} > K_{out}$), 0 added K^+, containing in addition 5 μg/ml valinomycin.

or absence of valinomycin. Transport was not evident with inwardly directed K_2SO_4 gradients. When K_2SO_4 gradients were outwardly directed however, a small amount of H^+ transport was observed, and valinomycin greatly increased the rate of H^+ transport. With outwardly directed K^+ gradients and valinomycin, rates of transport were similar to those observed with Cl^-. This Cl^- independent, K^+-valinomycin-stimulated H^+ transport activity was inhibited by diethylstilbesterol, *N*-ethylmaleimide, and by photolysis with 8-azido-ATP (not shown), suggesting that these agents inhibit the pump, rather than simply the associated Cl^- transport pathway upon which transport also depends. Thus, the role of Cl^- in lysosomal proton transport is simply to provide an adequate conductance.

Discussion

H^+-transporting ATPases, distinct from the mitochondrial and chloroplast H^+-translocating ATPases have been described in plants [24], in yeast [42], and in various mammalian membranes. They are located both in the plasma membrane and in intracellular organelles.

Delineation of the similarities and molecular differences in the H^+ pumps must probably await the purification of the pumps from the various organelles, including the lysosome, as has been recently achieved for the clathrin-coated vesicle pump [27] and the chromaffin granule membrane [28].

To determine proton pump purification, *N*-ethylmaleimide-sensitive H^+ pump and ATPase activities were determined at each step of the purification procedure. While the bovine brain clathrin-coated vesicle ATPase and H^+-pumps were uniformly sensitive to *N*-ethylmaleimide [27], the highly purified chromaffin-granule pump showed a high affinity site for *N*-ethylmaleimide in H^+ pump inhibition, and a low affinity site for ATPase inhibition. Similarly, lysosomal proton pump activity was sensitive to low levels of *N*-ethylmaleimide, but an alkaline ATPase activity of the lysosomal membrane shows a low sensitivity to *N*-ethylmaleimide [12]. Distinction between the possibility that these differences in *N*-ethylmaleimide sensitivity arises from the presence of more than one ATPase, one of which is related to proton transport, and the possibility that there exists a single ATPase in the membrane with two sites for inhibition is difficult to determine at this time. DCCD was employed with success in the chromaffin granule purification procedure to further identify the protein responsible for H^+ transport [28]. Unfortunately, this agent is apparently not uniformly useful for proton pumps, as exemplified by the fact that DCCD appears to abolish proton transport at 25 μM in lysosomes [12], but part of the inhibitory effects on lysosomal H^+ transport appear to result from alteration of membrane properties, rather than by reaction with the proton pump alone [13]. As a further complication, the yeast proton pump has been partially purified away from the yeast DCCD binding proteins [42].

Thus, it was necessary to investigate other potential inhibitors of the lysosomal H^+ pump and lysosomal ATPase that could be used in conjunction with the aforementioned inhibitors and others. Diethylstilbesterol is an inhibitor of several H^+ pumps. Diethylstilbesterol was also found to be a potent inhibitor of the lysosomal Mg^{2+}-stimulated ATPase and the ATP-dependent lysosomal H^+ pump, without affecting the membrane permeability as measured by a lack of effect upon H^+ leak rate and upon ATP-independent $DiSC_3(5)$ uptake. Similarly, photolysis with 8-azido-ATP resulted in an inhibition of the H^+ pump and Mg^{2+}-ATPase activity. A finite number of lysosomal membrane proteins were labelled using 8-azido-[α-^{32}P]ATP, suggesting that it might be possible to employ this tag to identify the ATP binding subunit of the lysosomal proton pump.

An additional important consideration in preparation for attempts at the isolation, purification, and identification of the function of the subunits of the lysosomal H^+ pump was the delineation of the role of the lysosomal membrane anion permeability in proton pump function. Externally added Cl^- appears to promote proton transport by a variety of pumps. Bennett and Spanswick showed that the corn root H^+ pump exhibited an absolute requirement for Cl^- [24], while the bovine clathrin-coated vesicle pump could function in the absence of added Cl^-. An examination of the role of Cl^- was therefore undertaken. A Cl^- conductance pathway was demonstrated using potential-sensitive dyes and the magnitude of the conductance pathway was estimated by following ATP-independent HCl accumulation through the Cl^- conductance. In a previous study of the clathrin-coated vesicle pump [21], valinomycin was used to facilitate loading of K^+. Since valinomycin was used for the loading process, and since valinomycin requires Cl^- for effective K^+ movement [21], it was not possible to rule out the effect of Cl^- on loading from the effect of Cl^- on pump function. In this study however, we found that near maximal rates of transport were obtained in the absence of Cl^-, as long as valinomycin was present following establishment of an outwardly directed K^+ gradient. The loading procedure employed here did not involve the use of valinomycin, and thus Cl^- was found not to be an obligate participant in the transport process.

Acknowledgement

Supported by NIH AM 17328, NIH DK 38808 and the Veterans Administration.

References

1 Njus, D. and Radda, G.K. (1978) Biochim. Biophys. Acta 463, 219–244
2 Codon, S. and Nelson, N. (1983) J. Biol. Chem. 256, 3858–3864
3 Zhang-F. and Schneider, R.L. (1983) Biochem. Biophys. Res. Commun. 114, 620–623
4 Glickman, J., Croen, K., Kelly, S. and Al-Awqati, Q. (1983) J. Cell Biol. 97, 1303–1308
5 Forgac, M., Cantley, L., Weidenmann, B., Altztiel, L. and Branton, D. (1983) Proc. Nat. Acad. Sci. USA 80, 1300–1303
6 Stone, D.K., Xie, X. and Racker, E. (1983) J. Biol. Chem. 258, 4059–4062
7 Ohkuma, S. and Poole, B. (1978) Proc. Natl. Acad. Sci. USA 75, 3327–3331
8 Mego, J.L., Farb, R.M. and Barnes, J. (1972) Biochem. J. 128, 763–769
9 Schneider, D.L. (1974) Biochem. Biophys. Res. Commun. 87, 559–565
10 Ohkuma, S., Moryama, Y. and Takano, T. (1982) Proc. Natl. Acad. Sci. USA 79, 2758–2762
11 Schneider, D.L. (1981) J. Biol. Chem. 256, 3858–3864
12 Moryama, Y., Takano, T. and Ohkuma, S. (1984) Biochem. J. 95, 995–1007
13 Harikumar, P. and Reeves, J.P. (1984) J. Biol. Chem. 258, 10403–10410
14 Schneider, D.L., Barnside, J., Gorga, F.R. and Nettleton, C.J. (1978) Biochem. J. 176, 75–82
15 Chung, C.-H., Elliot, R.L. and Mego, J.L. (1980) Arch. Biochem. Biophys. 322, 251–259
16 Reggio, H., Bainton, D., Harms, E., Coudrier, E. and Louvard, D. (1984) J. Cell Biol. 99, 1151–1526
17 Mollinedo, F. ad Schneider, D.L. (1984) J. Biol. Chem. 259, 7143–7150
18 Apps, D.K., Pryde, J.G., Sutton, R. and Phillips, J.H. (1980) Biochem. J. 190, 273–282
19 Van Dyke, R.W., Scharschmidt, B.F. and Steer, C.J. (1985) Biochim. Biophys. Acta 312, 423–436
20 Xie, X.-S. and Stone, D.K. (1986) J. Biol. Chem. 261, 2492–2495
21 Xie, X.-S., Stone, D.K. and Racker, E. (1983) J. Biol. Chem. 258, 14834–14838
22 Stone, D.K., Xie, X.-S. and Racker, E. (1984) J. Biol. Chem. 259, 2701–2703
23 Forgac, M. and Cantley, L. (1984) J. Biol. Chem. 259, 8101–8105
24 Bennett, A.B. and Spanswick, R.M. (1983) J. Membr. Biol. 71, 95–1071
25 Van Dyke, R.W., Steer, C.W. and Scharsmidt, B.F. (1984) Proc. Natl. Acad. Sci. USA 81, 3108–3112
26 Schneider, D.L. (1983) J. Biol. Chem. 258, 1833–1838
27 Xie, X.-S. and Stone, D.K. (1986) J. Biol. Chem. 261, 2492–2495
28 Cidon, S. and Nelson, N. (1986) J. Biol. Chem. 261, 9222–9227
29 Mitchell, P. (1966) Biol. Rev. (Cambridge) 41, 445–502
30 Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J.W., Fowler, S. and De Duve, C. (1968) J. Cell Biol. 37, 482–513
31 Trouet, A. (1974) Methods Enzymol. 31, 323–329
32 Worwood, M., Dodgson, K.S., Hook, G.E.R. and Rose, F.A. (1973) Biochem. J. 134, 183–190
33 Torriani, A. (1960) Biochim. Biophys. Acta 38, 460–469
34 Pennington, R.J. (1961) Biochem. J. 80, 649–654
35 King, T.E. (1963) J. Biol. Chem. 238, 4037–4051
36 Yoda, A. and Hokin, L.E. (1970) Biochem. Biophys. Res. Commun. 40, 880–886
37 Lee, H.C., Forte, J.G. and Epel, D. (1982) in Intracellular pH, Its Measurement, Regulation, and Utilization in Cellular Functions (Nucitelli, R. and Deamer, D.W., eds.), pp. 136–158, Alan R. Liss, New York
38 Haley, B.E. and Hoffman, J.F. (1974) Proc. Natl. Acad. Sci. USA 71, 3367–3371
39 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
40 Laemmli, U.K. (1970) Nature (Lond.) 227, 680–685
41 Marin, B., Marin-Lanza, M. and Komor, E. (1981) Biochem. J. 198, 365–372
42 Uchida, F., Oshumi, Y. and Naraku, Y. (1985) J. Biol. Chem. 260, 1090–1095
43 Miller, T.M. and Heuser, J.E. (1984) J. Cell Biol. 98, 685–698
44 Sherman, D., Nordman, J. and Henry, J.-P. (1982) Biochemistry 21, 687–694
45 Borst-Pauwels, G.W.F.H., Theuvenet, A.P.R. and Stols, A.L.H. (1983) Biochim. Biophys. Acta 732, 186–192
46 Gluck, S., Kelly, S. and Al-Awqati, Q. (1982) J. Biol. Chem. 257, 9230–9233