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Characterization of a vacuolar proton ATPase in *Dictyostelium discoideum*

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Of the total ATPase activity in homogenates of the ameba, *Dictyostelium discoideum*, approximately one-third was inhibited at pH 7 by 25 μM 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl). Upon isopycnic sucrose density gradient centrifugation, the bulk of the NBD-Cl-sensitive ATPase activity was recovered in a major membrane fraction with a broad peak at 1.16 g/ml, well-resolved from markers for plasma membranes, mitochondria, lysosomes and contractile vacuoles. The gradient peak had a specific activity of 0.5 μmol/min per mg protein. The activity was half-inhibited by 1 μM silicotungstate, 2 μM diisothiocyanatostilbene disulfonate (DIDS), 2.5 μM dicyclohexylcarbodiimide (DCCD), 4 μM NBD-Cl and 20 μM *N*-ethylmaleimide (NEM) but was resistant to conventional inhibitors of mitochondrial and plasma membrane ATPases. That this ATPase activity constituted a proton pump was shown by the MgATP-dependent uptake and quenching of Acridine orange fluorescence by partially purified vacuoles. The Acridine orange uptake was specifically blocked by the aforementioned inhibitors. The generation of proton electrochemical gradients was suggested by the stimulation of enzyme activity by protonophores (fatty acids) and cation exchangers (nigericin). Uncoupling stimulated the ATPase activity as much as 20-fold, revealing an unusually high impermeability of the membranes to protons. ATPase activity was also stimulated by halide ions, apparently through a parallel conductance pathway. Under a variety of sensitive test conditions, the reverse enzyme reaction (i.e., incorporation of $^{32}P_i$ into ATP) was not detected. We conclude that this major H^+-ATPase serves to acidify the abundant prelysosomal vacuoles found in *D. discoideum* (Padh et al. (1989) J. Cell Biol. 108, 865–874). The finding of a vacuolar H^+-ATPase in a protist suggests the ubiquity of this enzyme among the eukaryotic kingdoms.

Introduction

Vacuolar H^+-ATPase is a unifying feature of a class of diverse organelles which have in common the transfer of their membranes and acidified contents through intermittent fusion and fission. Among these organelles are yeast vacuoles [1], plant tonoplasts [2], clathrin-coated vesicles [3,4], secretory granules [5,6], the *trans* Golgi apparatus [7], lysosomes [8], the compartment of uncoupling of receptor and ligand (CURL; Refs. 9 and 10) and prelysosomal processing vacuoles in, for example, *Paramecium* and mammalian kidney [11,12]. Because the organisms examined in these studies represent a wide range of phyla, and because the vacuolar ATPase appears to be structurally and genetically related to prokaryotic F_1F_0-ATPases (cf. Ref. 13), it is tempting to suppose that this enzyme is universal among eukaryotes. However, Protista, the eukaryotic kingdom which diverged earliest, has not been examined in this regard.

We have recently demonstrated the presence of abundant acidic prelysosomal vacuoles in the ameba, *Dictyostelium discoideum* [14]. We now show that these vacuoles have a highly active proton pump which closely resembles that found in higher eukaryotes.

Materials and Methods

Materials

The following biochemicals were obtained from Sigma Chemicals: ascorbic acid, NEM, NBD-Cl, EDAC, Mes, Acridine orange, thimerosal, oligomycin, soybean phosphatidylcholine (type III-S), bovine brain phosphatidylserine, dithiothreitol, and sodium azide. We

Abbreviations: Ado*PP*[NH]*P*, 5′-adenylyl imidodiphosphate; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Mes, 4-morpholineethanesulfonic acid; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; NEM, *N*-ethylmaleimide.

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purchased DCCD and DIDS from Pierce. Sodium vanadate and oleic acid were from Fisher and nigericin was from Calbiochem. Polycarbonate filters (25 mm in diameter with 5 μm pores) were obtained from Nucleopore and silicotungstic acid (Mol. Wt. 3310) from Polysciences. Other reagents were at least of analytical grade.

D. discoideum, strain Ax-3, was cultured and prepared as described in Ref. 14.

Isolation of H^+-ATPase-rich vacuoles

Cells were homogenized in 5 mM sodium-glycinate (pH 8.5) by forcing them through a pair of polycarbonate filters with 5 μm pores as described [15]. The extent of homogenization was monitored by dark-field microscopy and was usually judged to be complete.

From analytical isopycnic fractionation experiments such as that shown in Fig. 1, we have established a simple procedure to partially purify the buoyant acidic vacuoles from the denser organelles. Fresh homogenates were immediately adjusted to a sucrose concentration of 38% (w/w) and spun at 15000 rpm in a Sorvall SS-34 rotor for 1 h. The pellets were used as the source of mitochondrial H^+-ATPase. The supernatants were diluted with the sodium glycinate buffer to 10% sucrose and spun in the SS-34 rotor at 15000 rpm for 20 min. The resulting pellet contained most of the NBD-Cl-sensitive ATPase activity but scarcely any of the mitochondrial, lysosomal, plasma membrane or soluble markers. This fraction was used as the source of the acidic vacuole H^+-ATPase in experiments other than that in Fig. 1.

Assays

ATPase activity was determined by the optical absorbance of the phosphomolybdate complex at 750 nm, as described in Ref. 14. Unless otherwise indicated, the reaction contained (in 350 μl) 10–50 μg membrane protein in 5 mM $MgCl_2$ buffered with either 25 mM imidazole-HCl or a mixture of 50 mM Tris plus 50 mM Mes at pH 7.0. Reaction mixtures were preincubated at 30°C for 5 min before the addition of ATP to 2 mM. After 10 min incubation at 30°C, the reaction was terminated by the addition of 0.7 ml of 2% H_2SO_4 containing 0.5% sodium dodecylsulfate and 0.5% ammonium molybdate, followed by 10 μl of 10% ascorbic acid. Since micromolar NBD-Cl is considered to be a selective inhibitor of vacuolar H^+-ATPase [2,9], this enzyme was defined operationally by the difference in the rate of ATP hydrolysis in the presence and absence of 25 μM NBD-Cl at pH 7.0. The presence of Tris or imidazole plus Cl^- fostered maximal activity and minimal stimulation by the uncoupler, nigericin. For maximum coupling, Tris and chloride were replaced by Mes and sulfate and the temperature was lowered to 15°C to slow the loss of coupling (see Results and Discussion and Fig. 5). For assay of mitochondrial H^+-ATPase, Tris-Mes was replaced by 50 mM sodium glycinate (pH 9.5).

Succinate dehydrogenase [16] was assayed in 1 ml of 100 mM potassium phosphate (pH 7.0)/13 mM sodium succinate/10 mM KCl/1 mM $K_3Fe(CN)_6$. The rate of decrease in absorbance was monitored at 400 nm.

Alkaline phosphatase [17], acid phosphatase [18], alkaline phosphodiesterase [19] and *N*-acetyl-D-glucosaminidase [18] reactions were carried out in a volume of 250 μl at 20°C for 30 min. The following buffers and substrates were used. Alkaline phosphatase: 100 mM Tris-borate (pH 8.5)/15 mM $MgCl_2$/15 μM $ZnSO_4$/0.2% Triton X-100 with 10 mM *p*-nitrophenyl phosphate as substrate. Acid phosphatase: 100 mM glycine-HCl (pH 3)/0.2% Triton X-100 with 10 mM *p*-nitrophenyl phosphate as substrate. Alkaline phosphodiesterase: 100 mM Tris-borate (pH 8.5)/8 mM $MgCl_2$/8 μM $ZnSO_4$/0.2% Triton X-100 with 10 mM *p*-nitrophenylthymidine 5′-phosphate as substrate. *N*-Acetyl-D-glucosaminidase: 100 mM glycine acetate (pH 4.5)/1 mM $MgCl_2$/0.2% Triton X-100 with 4 mM *p*-nitrophenyl-*N*-acetyl-D-glucosaminide as substrate. The reactions were terminated with 750 μl of ice-cold 0.2 M Na_3PO_4 (pH 12). The *p*-nitrophenol liberated was estimated by its absorbance at 410 nm.

Protein was determined by the method of Bradford [20].

Results and Discussion

Subcellular fractionation of ATPase activity

Approximately one-third of the total cellular ATPase in the homogenate of *D. discoideum* at pH 7.0 was inhibited by 25 μM NBD-Cl. The specific activity of the NBD-Cl-sensitive component was roughly 0.1 μmol/min per mg homogenate protein. No higher specific cellular content has thus far been reported for an NBD-Cl-sensitive ATPase activity.

The NBD-Cl-sensitive ATPase activity had a broad and complex density profile upon isopycnic centrifugation in sucrose (Fig. 1A). The major peak of activity was found at 1.16 g/ml; it had a maximal specific activity of approx. 0.5 μmol/min per mg protein and coincided with a distinct peak of protein (Fig. 1D). Because the ATPase activity in this buoyant peak was almost entirely inhibited by NBD-Cl, we infer that these membranes contained essentially only one form of the enzyme. This activity has the properties of a vacuolar H^+-ATPase [21–23] and will be referred to as such.

Homogenates of *D. discoideum* contained at least three other classes of ATPase activity:

(a) Soluble ATPases were found at the top of gradients (right side of the profile in Fig. 1A). They had an overall specific activity of 0.015 μmol/min per mg

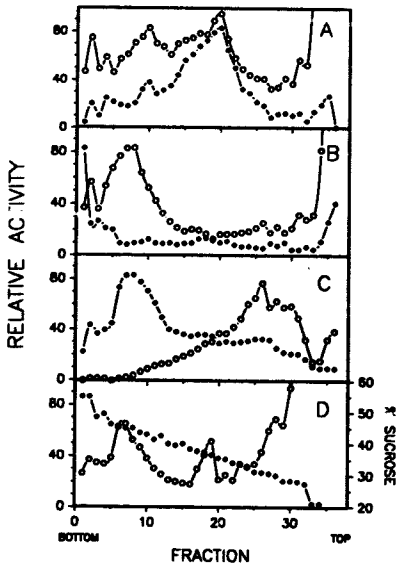

Fig. 1. Fractionation of *D. discoideum* organelles by sucrose density gradient equilibrium centrifugation. Amebae from 120 ml culture were washed and resuspended in ice-cold growth medium at $8 \cdot 10^7$ ml^{-1}. To label the plasma membranes, 2 μCi of [^{3}H]cholesterol in 20 μl ethanol was added to 10 ml of cell suspension [34]. After rocking for 20 min in the cold, the cells were washed twice, resuspended in 4 ml of ice-cold 5 mM sodium glycinate (pH 8.5) containing 100 mM sucrose and homogenized by forcing them through a pair of polycarbonate filters [15]. 2.5 ml of the homogenate was layered on a 34 ml of a linear gradient of 25–45% sucrose (w/w) on a 2 ml cushion of 55% sucrose (w/w), all in sodium glycinate buffer. After centrifugation in a Beckman SW-27 rotor at 25000 rpm for 3 h at 1°C, 1-ml fractions were collected from the bottom of the tube and assayed for the markers indicated. Panel A: total (○) and NBD-Cl-sensitive (●) ATPase activity at pH 7.0. Panel B: acid phosphatase (○) and mitochondrial ATPase (assayed at pH 9.5) (●). Panel C: alkaline phosphodiesterase (○) and [^{3}H]cholesterol (●). Panel D: protein (○) and gradient sucrose (●). The high protein concentration and total ATPase activity in the very top (soluble) fractions of the gradient were not plotted.

protein and were not sensitive to NBD-Cl. They were not further characterized.

(b) The mitochondrial H^+-ATPase was identified by its similarity to other F_1F_0-ATPases [21–23]. It had a pH optimum of 9.5, was half-inhibited by 0.1 μg/ml oligomycin, pelleted at 10^5 $g \cdot$ min and equilibrated on sucrose density gradients along with succinate dehydrogenase at 1.22 g/ml (Fig. 1B). Its specific activity at pH 9.5 in the gradient peak was 0.4 μmol/min per mg protein. However, at the pH 7 used in the assay of the vacuolar ATPase, its specific activity was only 0.06 μmol/min per mg protein (Fig. 1A). There was less protein in the mitochondrial fraction than in the buoyant vacuole peak (Fig. 1D).

(c) Approx. 40% of the ATPase activity in the homogenate at pH 7 was inhibited by 100 μM vanadate. Roughly half of this activity was not sedimentable, while most of the rest had an isopycnic density of 1.20 g/ml on sucrose density gradients corresponding to the large peak at fraction 10 in Fig. 1A. This distribution resembled that of exogenous surface labels, such as [^{3}H]cholesterol (compare Figs. 1A and C). The peak specific activity of this ATPase was 0.3 μmol/min per mg protein, and half-inhibition by vanadate occurred at 10 μM. These data are all consistent with the presence of a cell surface vanadate-sensitive H^+-ATPase in *D. discoideum* (see Refs. 21, 23–25, 27).

For reference, we also demonstrated that alkaline phosphodiesterase activity distributed in parallel with alkaline phosphatase activity at 1.14 g/ml (Fig. 1C). We take these two activities to be markers for contractile vacuoles [26].

Acid phosphatase and acid *N*-acetyl-glucosaminidase equilibrated at 1.21 g/ml (Fig. 1B) and were ascribed to lysosomes [18]. Lysosomes may have been the source of the minor and variable NBD-Cl-sensitive ATPase activity found at high density (Fig. 1A). Under our homogenization conditions, most of each acid hydrolase activity was pelletable; therefore, the buoyant H^+-ATPase could not have been derived from broken lysosomal membranes.

Because so little of the NBD-Cl-sensitive ATPase activity was found in fractions rich in soluble protein, mitochondria, plasma membranes, contractile vacuoles and even lysosomes, this enzyme appears to be rather characteristic of the buoyant membranes. Since we have detected only one major peak of NBD-Cl-sensitive membrane ATPase in our homogenates, it is likely to be the proton pump of the acidic vacuoles described earlier [14].

Facile preparation of buoyant vacuoles

The NBD-Cl-sensitive ATPase sedimented readily in the Sorvall SS-34 rotor; half of the activity in the homogenate was cleared from the supernatant by 50000 $g \cdot$ min and 90% by 400000 $g \cdot$ min. Based on the sedimentability and buoyancy of these membranes (Fig. 1A), we devised a simple method for their partial purification. Cell homogenates were brought to a density of 1.17 g/ml in 38% sucrose (w/w) and centrifuged to pellet the dense nuclei, mitochondria, lysosomes and plasma membranes. The supernatant fraction was diluted to a density of 1.04 g/ml and recentrifuged to separate the acidic vacuole membranes from soluble contaminants (see Materials and Methods). The pelleted membranes typically contained 70% of the vacuolar ATPase of the homogenate but only 10–20% of the mitochondrial and plasma membrane ATPases described above. The NBD-Cl-sensitive ATPase in the buoyant membranes had a specific activity of approx. 0.5 μmol/min per mg protein, similar to that obtained from continuous analytical isopycnic gradients. It is noteworthy that the specific activity of this vacuolar

H^+-ATPase is 10–35-fold greater than that reported for purified coated vesicles [3,4], 11-fold greater than that of purified chromaffin granules [6], and at least as great as that of yeast vacuoles [1] and tonoplast membranes [2].

Inhibitor studies

The ATPase activity in the buoyant vacuole fraction was inhibited 80–90% by two sulfhydryl reagents (Figs. 2A and B). The residual 10–20% ATPase activity remaining at saturation probably reflects contamination by plasma membranes, since it was sensitive to vanadate inhibition. The action of other inhibitors is also illustrated in Figs. 2 and 3. The inhibition curves were also hyperbolic; half-inhibition was observed at these micromolar concentrations: silicotungstate, 1; DIDS, 2; DCCD, 2.5; NBD-Cl, 4; thimerosal, 5; and NEM, 20. The vacuolar ATPase activity was insensitive to oligomycin (Fig. 3A), to a hydrophilic carbodiimide, EDAC (Fig. 3B), and to 0.1 mM vanadate, 0.1 mM azide and 1 mM ouabain (data not shown). This pattern of inhibition is that found generally for vacuolar H^+-ATPases in higher organisms [9,13,21,23,27]. In contrast, azide and DCCD as well as oligomycin (Fig. 3A) all inhibited the dense, alkaline ATPase which clearly corresponds to mitochondria [2,21,23,27,29].

Silicotungstate has been shown to inhibit ATPases of the F_1 type [28]. It was a poor inhibitor of mitochondrial F_1-ATPase in our system (Fig. 2D), perhaps because of the impermeability of our intact mitochondrial preparations. It was noteworthy that silicotungstate was a far more potent inhibitor of the NBD-Cl-sensitive ATPase in *D. discoideum* ($K_{1/2} = 1$ μM; Fig. 2C) than the F_1-ATPase in bovine heart submitochondrial particles ($K_{1/2} = 15$ μM; Ref. 28). Sensitivity to silicotungstate can be added to the growing documentation of the structural, functional and evolutionary relationship of vacuolar to F_1-ATPases [13].

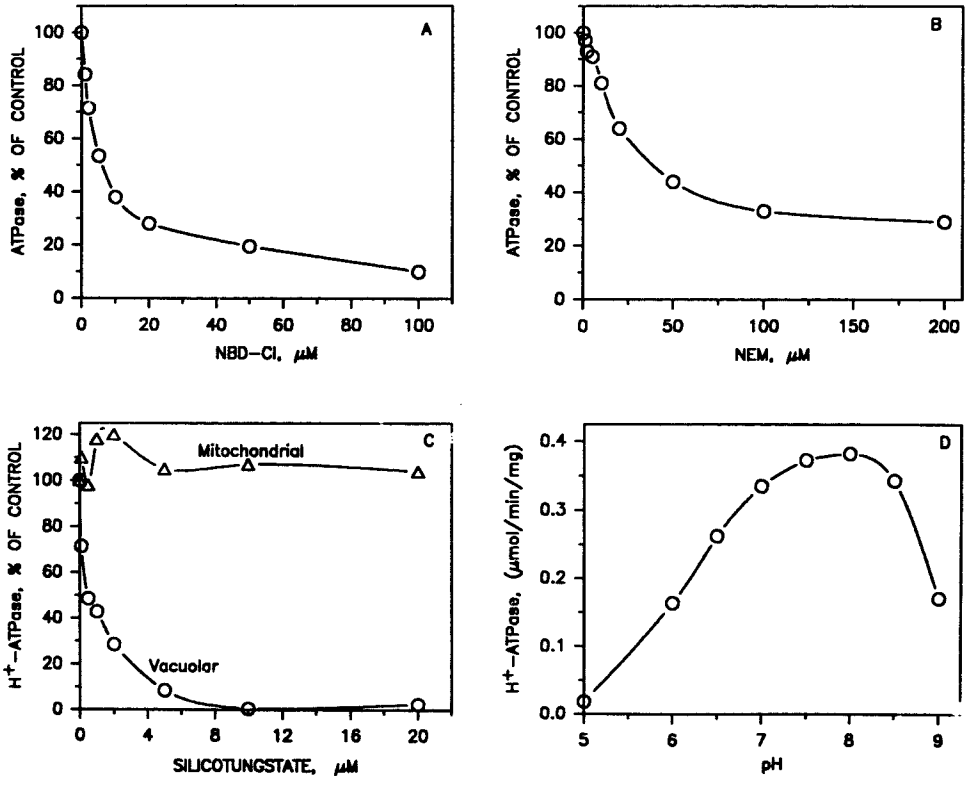

Fig. 2. Characterization of the vacuolar ATPase. Panel A: NBD-Cl sensitivity. Buoyant vacuoles (13 μg protein) were preincubated at 30 °C for 5 min with the indicated concentration of NBD-Cl in 350 μl before the ATPase reaction was initiated by addition of 2 mM ATP (see Materials and Methods). The uninhibited specific activity was 0.5 μmol/min per mg protein. Panel B: NEM sensitivity. As in Panel A, except that NEM and 15 μg vacuole protein were used. Panel C: silicotungstate sensitivity. The 350 μl assays contained 20 mM KCl/5 mM $MgCl_2$/25 mM imidazole-HCl (pH 7.0) ± 25 μM NBD-Cl for uncoupled vacuolar ATPase or 50 mM glycine-NaOH (pH 9.5) plus 5 mM $MgCl_2$ for mitochondrial ATPase activity plus 10 μg protein from the respective fractions. The uninhibited specific activities were 0.35 and 0.38 μmol/min per mg protein for the vacuolar and mitochondrial ATPases, respectively. Panel D: effect of pH. As in Panel A except that the reactions were in 1 ml and contained 33 μg protein ± 25 μM NBD-Cl in 50 mM Tris-Mes buffer at the indicated pH.

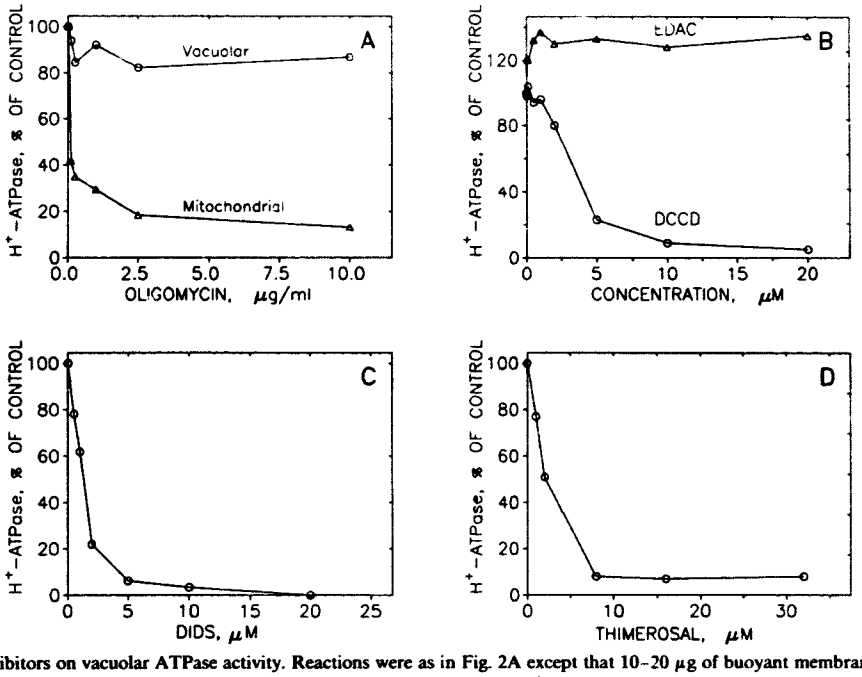

Fig. 3. Effect of inhibitors on vacuolar ATPase activity. Reactions were as in Fig. 2A except that 10–20 μg of buoyant membrane protein ± 25 μM NBD-Cl was used. Panel A: oligomycin. The effect of oligomycin on mitochondrial H^+-ATPase from the dense membrane fraction is also shown; this assay was performed in 50 mM sodium glycinate (pH 9.5). Panel B: DCCD and EDAC. Panel C: DIDS. Panel D: thimerosal. Uninhibited activities were 0.4–0.6 and 0.35 μmol/min per mg protein for the vacuolar and the mitochondrial H^+-ATPase, respectively.

Other properties of the vacuolar ATPase

The enzyme had an optimal activity near pH 8.0 (Fig. 2D). We nevertheless performed the assay at pH 7 to minimize the expression of mitochondrial H^+-ATPase activity. The dependence of this activity on MgATP was hyperbolic with a K_m of 200 μM and a V_{max} of 0.52 μmol/min per mg protein (Fig. 4). The activity of the enzyme varied with nucleoside triphosphate substrates

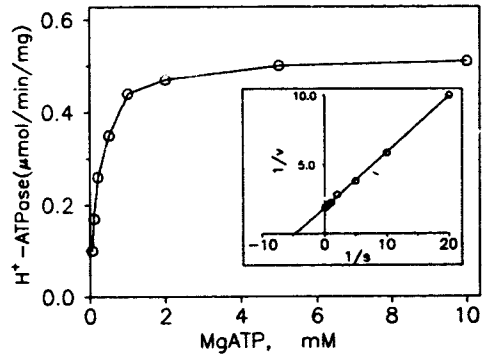

Fig. 4. Dependence of NBD-Cl-sensitive ATPase activity on MgATP. Reaction mixtures containing 10 μg of buoyant membrane protein in 50 mM Tris-Mes (pH 7.0) and 20 mM NaCl were preincubated and the reaction then initiated by the addition of indicated equimolar mixture of $MgCl_2$ plus ATP. Inset: double-reciprocal plot giving a K_m of 200 μM and V_{max} of 0.52 μmol/min per mg protein.

TABLE I

Inactivation and reactivation of the vacuolar ATPase by Triton X-100

Lecithin was dried from chloroform under a nitrogen stream and redispersed by vigorous vortexing in 50 mM Tris-Mes (pH 7) containing 5 mM $MgCl_2$. Bio-Beads SM-2 were washed six times with water and resuspended to a 50% (v/v) suspension in H_2O. The buoyant membrane fraction described in Materials and Methods was suspended in the stated buffer. Triton X-100 was added to aliquots of the membranes to a final concentration of 0.04% followed by incubation at 4°C for 10 min. Lecithin was then added to aliquots which were incubated further at 24°C for 10 min. This was followed by the addition of Bio-Beads SM-2 to 0.28 ml/ml to the indicated tubes. The tubes were all incubated at 4°C with gentle rocking for 30 min. The beads were removed at unit gravity, and the ATPase in the supernatant was assayed in the absence or presence of 25 μM NBD-Cl following the addition of 2 mM ATP as outlined in Materials and Methods. All samples had the same final buffer concentration and 30 μg/ml of membrane protein in a final volume of 350 μl.

Addition	H^+-ATPase activity (μmol/min per mg protein)
None	0.49
Triton X-100	0.07
Lecithin (130 μg/ml)	0.49
Triton X-100, then lecithin (130 μg/ml)	0.26
Lecithin (434 μg/ml)	0.39
Triton X-100, then lecithin (434 μg/ml)	0.30
Bio-Beads alone	0.26
Triton X-100, then Bio-Beads	0.28
Triton X-100, then lecithin (434 μg/ml), then Bio-Beads	0.26

in this order: A > G > U > C > T. Ado*PP*[NH]*P* and ADP were not hydrolyzed, but they were potent inhibitors. AMP was neither a substrate nor an inhibitor. ATP and dithiothreitol were found to protect the enzyme from irreversible inactivation by NBD-Cl (data not shown). All of these characteristics resemble those of the vacuolar H^+-ATPases in higher organisms [9,21,23,27].

Effect of Triton X-100 on the vacuolar ATPase

H^+-ATPase activity was strongly inhibited by 0.04% Triton X-100. The subsequent addition of Bio-Beads SM-2 restored its activity, as did an excess of phospholipids (Table I). In other experiments, 0.09% Triton X-100 converted the enzyme to an inactive form which was not sedimented by centrifugation for $5 \cdot 10^6$ $g \cdot$ min. Treatment of the inactive supernatant fraction following centrifugation with Bio-Beads SM-2 restored approx. 60% of the initial activity in a sedimentable form. It is likely that reactivation signifies reintegration of solubilized enzyme in the membrane.

Coupling of the NBD-Cl-sensitive ATPase to the electrochemical proton gradient

Fig. 5 summarizes evidence that the H^+-ATPase activity under study works against a membrane electrochemical gradient for protons, as is the case for other proton pumps [21,23,27]. Curve A demonstrates the activation of the enzyme by KCl in a coupling buffer. As seen in the comparison of curves A and E, the high activity was dependent on the presence of the cation

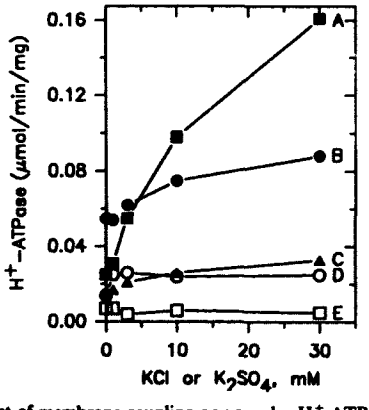

Fig. 5. Effect of membrane coupling on vacuolar H^+-ATPase activity. Buoyant vacuoles (20 μg protein) were mixed with coupling buffer (25 mM Mes-NaOH (pH 7)/5 mM K_2SO_4/5 mM $MgSO_4$) containing salt solutions, phospholipids and 5 μM nigericin as indicated. The assay was conducted in 350 μl ± 25 μM NBD-Cl. The phospholipids were 150 μg/assay of a 3:1 mixture of phosphatidylcholine and phosphatidylserine. The assays were as described except at 15°C. The mixtures contained: curve A: KCl, phospholipids and nigericin; B, KCl and nigericin without phospholipids; C, K_2SO_4, phospholipids and nigericin; D, KCl without phospholipids or nigericin; E, KCl and phospholipids without nigericin.

exchanger, nigericin, in the presence of a transportable cation, K^+. Valinomycin and oleic acid could substitute for nigericin plus K^+ (data not shown). Stimulation was as much as 20-fold, 5–10-times greater than observed with other vacuolar H^+-ATPase preparations (see, for example, Ref. 3). The weak bases, Tris, imidazole and ammonium (unlike Na^+ or K^+) also caused activation, while zwitterionic buffers (such as the Mes buffer used here) did not. The effects of nigericin and the organic amines were not additive; we suggest that they all acted by reducing the pH gradient across the membrane.

Curve B reveals that the dependence on $[Cl^-]$ is relieved by the omission of exogenous phospholipids from the incubation mixture. We suggest that Cl^- might act by dissipating a membrane potential and that exogenous phospholipids might act as a sink which removes endogenous fatty acid uncouplers.

Curve C shows that K_2SO_4 does not substitute for KCl. Related studies showed that Br^- did substitute for Cl^- but that borate and acetate did not. Cl^--dependence could signify its direct activation of the enzyme or HCl cotransport. However, neither of these possibilities is consistent with the observation of high H^+-ATPase activity in the absence of halide ions when phospholipids are omitted or oleic acid is added as a protonophore (data not shown). An alternative mechanism is the operation of a parallel electrogenic anion transporter [30]. In our view, the inhibition of vacuolar H^+-ATPase activity by DIDS (seen here in Fig. 3C) is not related to dissipation of electrical gradients by anion transport, because the inhibition is not relieved by the protonophore, oleic acid (data not shown). Perhaps DIDS acts directly on the vacuolar ATPase. In any case, a parallel Cl^- conductance pathway is the mechanism most consistent with our data and that of others [30].

Curves D and E both demonstrate that ATPase activity was poor when nigericin was omitted. The difference between these curves shows that exogenous phospholipids typically suppress ATPase activity, presumably by increasing coupling.

H^+-ATPase reversal

Using the incorporation of $^{32}P_i$ into ATP as a criterion, we tested for the reverse or exchange reaction of the H^+-ATPase. We consistently failed to observe such an NBD-Cl- or nigericin-sensitive activity under a wide variety of tightly-coupled and uncoupled conditions. The limit of detection in our experiments was 20 pmol/min per mg protein, approx. 1 part in 25000 of the observed rate of ATP hydrolysis (namely, 0.5 μmol/min per mg protein).

In contrast to these findings, Stone et al. [31] have reported an exchange activity in coated vesicles of 860 pmol/min per mg protein, even though the ATPase activity of their preparations was 25-fold lower than in

ours. One explanation of this difference could be the dissipation of the proton gradient by a leak pathway in our membranes. However, our preparations showed unusually tight coupling, whereas coupling was not directly assessed by Stone et al. [31]. In any case, both our data and that of Stone et al. [31] support the premise that vacuolar H^+-ATPases are far less reversible than F_1-ATPases [22]. This behavior is consistent with the divergent roles of the two enzymes: the vacuolar H^+-ATPase uses ATP to acidify while F_1-ATPases harvest electrochemical gradients as ATP [21,22].

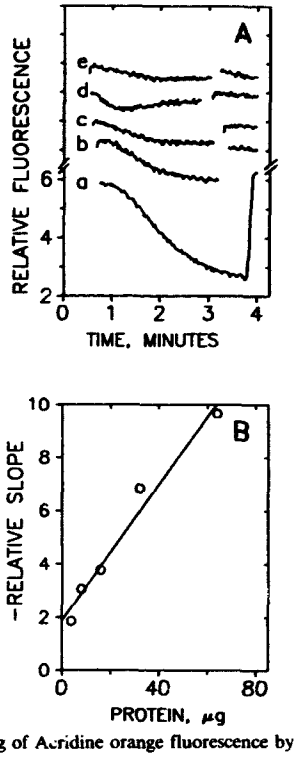

Fig. 6. Quenching of Acridine orange fluorescence by buoyant membranes. Panel A: each reaction mixture contained 30 mM KCl/10 mM NaCl/5 mM K_2SO_4/5 mM $MgSO_4$/5 mM sodium glycinate (pH 8.5)/0.1 mM EDTA/3 μM Acridine orange and 60 μg membrane protein in 2.1 ml. Individual mixtures were preincubated for 5 min in a cuvette at 15°C with the specified inhibitor and the reaction initiated by the addition of 1 mM ATP. The fluorescence was recorded in an Aminco-Bowman spectrofluorometer at 15°C using excitation and emission wavelengths of 480 and 530 nm. Fluorescence was continuously recorded by computer using Labtech Notebook software. Approx. 3–4 min after the addition of ATP, nigericin was added to 5 μM (shown by the interruption in the recording) and the recording continued. The subsequent tracings correspond in sequence to those on the left. Traces b–e have been arbitrarily displaced upward for legibility. Trace a: complete mixture; trace b: minus NaCl and KCl; trace c: plus 50 μM NBD-Cl; trace d: plus 10 μM DCCD; and trace e: plus 100 μM NEM. Panel B: a set of tracings was collected as in panel A, trace a, except that the membrane protein was varied. The slopes (rates of loss of Acridine orange fluorescence) were calculated for each 15 s segment of each trace. The maximum slope (steepest decline in fluorescence) occurred between 1.5 and 2.5 min; these maxima were plotted in arbitrary units against the respective protein concentration. The loss of Acridine orange fluorescence in the presence of membrane was shown to be completely and immediately reversed by addition of 5 μM nigericin in all cases. The slight loss of fluorescence in the absence of membrane was presumably due to photobleaching.

Demonstration of vacuole acidification in vitro

The buoyant membrane fraction caused a major reduction of the fluorescence of Acridine orange in a MgATP-dependent fashion (Fig. 6A). This effect was quantitative; that is, the initial rate of loss of fluorescence was directly proportional to the membrane concentration over a 15-fold range (Fig. 6B). That the loss of fluorescence was caused by the accumulation of the dye in an acidic membrane compartment was suggested by the complete and immediate reversal of the effect by nigericin and was confirmed by direct visualization of the large vacuoles in the fluorescence microscope (see Ref. 14). Acridine orange quenching was apparently mediated by the enzyme under investigation, since it was inhibited by NBD-Cl, NEM and DCCD (Fig. 6A) but not by vanadate, azide or oligomycin (data not shown). Furthermore, dye accumulation was Cl-dependent (trace b). Finally, we demonstrated the Acridine orange fluorescence quenching activity fractionated with the buoyant membranes containing the NBD-Cl-sensitive ATPase on sucrose density gradients (data not shown).

We frequently observed that the quenching of Acridine orange reversed gradually after 5–10 min of reaction and often nearly disappeared after 30 min. The agent causing this reversal was transferable to fresh vacuoles in the soluble supernatant obtained from incubated membranes. Certain precautions minimized the reversal; these included pre-washing the vacuoles with a phospholipid dispersion, lowering the assay temperature to 15°C, and the addition of 100 mM sucrose. We suspect that phospholipid hydrolysis may release fatty acids into the medium during the incubation, which then act as uncouplers. Adding exogenous phospholipids might then sequester the fatty acids and preserve coupling. Thus, under favorable conditions, we saw no evidence in these membranes for the high intrinsic proton permeability noted by Mellman et al. [10,32].

Concluding comments

We have demonstrated in *Dictyostelium* the presence of a membrane ATPase activity which resembles the vacuolar proton pumps found in several higher eukaryotic cells types (for reviews, see Refs. 9, 13, 21, 23 and 27). These similarities include: (a) Enzyme properties: pH optimum, MgATP-dependence, and nucleotide preference. (b) Insensitivity to vanadate (an inhibitor of acyl phosphate-type cation pumps) and oligomycin (an

inhibitor of F_1 H^+-ATPases). (c) Sensitivity to the inhibitors NBD-Cl, NEM, DCCD and DIDS. (d) Membrane association; including inhibition and solubilization by nonionic detergents and reactivation by phospholipids. (e) Coupling to transmembrane proton electrochemical gradients, as demonstrated by the stimulation of enzyme activity with cation exchangers and protonophores. (f) Stimulation by an apparent parallel Cl^- conductance pathway. (g) Undetectable reversal of and isotope-exchange by the H^+-ATPase. (h) MgATP-dependent acidification of isolated vacuoles. Unlike some other systems [32], our preparations were well-coupled to the membrane electrochemical gradient, as evidenced by more than 20-fold stimulation of activity in the presence of nigericin and Cl^- (Fig. 5).

These data add the ancestral kingdom of protists to those of fungi, plants and animals as bearers of cytoplasmic vacuole H^+-ATPase, establishing the ubiquity of this enzyme among eukaryotes. *Dictyostelium* is of particular interest because it diverged early in evolution, before yeasts, ciliates and even acanthamebae [33].

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