An electrogenic proton pump in plasma membranes from the cellular slime mould *Dictyostelium discoideum*

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Plants and fungi possess an outwardly directed plasma membrane proton pump that may regulate intracellular pH. We provide the first demonstration that amoebae of the slime mould *Dictyostelium discoideum* also possess a similar proton pump. It can be assayed either as an ATPase activity in highly purified plasma membranes or as a proton pump, after solubilization and reconstruction into liposomes. The pump is inhibited by vanadate, diethylstilbestrol (DES) and miconazole but not by azide or ouabain. The proton pump described here may represent the target for the action of DES and miconazole, both of which have previously been shown to induce stalk cell formation during the in vitro development of *Dictyostelium*.

Dictyostelium discoidem *Proton pump* *Mg^{2+}-ATPase* *Plasma membrane*

1. INTRODUCTION

During development of *Dictyostelium discoideum* the initially separate amoebae aggregate together and later the aggregate transforms into a fruiting body consisting of a cellular stalk supporting a mass of spores. Thus, an individual amoeba can develop into either a stalk or a spore cell and from indirect experiments, in which amoebae incubated in vitro were directed towards either of these fates, authors in [1] proposed that intracellular pH (pH_i) regulates this choice, with low pH_i favouring stalk cell differentiation.

In other free living eukaryotes such as yeast and fungi and also in plants, pH_i appears to be regulated in part by the activity of an outwardly directed, plasma membrane proton pump [2]. This enzyme is distinct from the mitochondrial proton translocating ATPase in its polypeptide structure, its resistance to azide inhibition and its sensitivity to sodium vanadate, DES and miconazole [3,4].

In work with *Dictyostelium* it was found that DES and miconazole can induce stalk cell differentiation, mimicking the action of the natural inducer DIF ([1] and unpublished). Vanadate was without effect, possibly due to a lack of uptake by the cells [5]. It therefore seemed likely that *Dictyostelium* possesses a fungal-type proton pump which normally maintains the cytoplasmic pH by secreting protons, and that when this pump is inhibited by DES or miconazole the resultant drop in pH_i induces stalk cell differentiation [1].

We show that *Dictyostelium* indeed possess a plasma membrane proton pump which can be assayed either as an ATPase or, after reconstitution into liposomes, by ATP-dependent pumping of protons. The pump is sensitive to vanadate, DES and miconazole but not to azide.

Abbreviations: ACMA: 9-amino-6-chloro-2-methoxy acridine; CCCP: carbonyl cyanide *m*-chlorophenylhydrazone; DES: diethylstilbestrol; DIF: differentiation inducing factor; I_{50}: inhibitor concentration giving 50% inhibition; PMSF: phenylmethylsulphonyl fluoride

2. MATERIALS AND METHODS

Asolectin (phosphatidylcholine) was from Associated Concentrates, NY.

ACMA was a generous gift from R. Kraayenhof

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(Free University, Amsterdam). ATP and GTP disodium salts were from Boehringer, CHAPS, CHAPSO and Iodogen from Pierce, Zwittergents Z-8 –Z-16 from Calbiochem and lysolecithin (Type I), CTP IV, GTP II-S, UTP III, ADP, AMP, CCCP, valinomycin and Mg-ATP from Sigma. Other commercial nucleotide preparations were inferior substrates.

2.1. *Cell growth*

Strain Ax 2 was grown axenically in flasks [6] or in 10 l aspirators as described [7] except that a more acidic medium was used: 114 g bacterial peptone (Oxoid), 57 g yeast extract (Oxoid), 1.4 g NaCl, 0.6 g KCl and 8 ml orthophosphoric acid were dissolved in 8 l and brought to pH 5.0 with KOH. After autoclaving 123 g of sterile glucose in 500 ml H_2O and 0.9 ml antifoam A (Sigma) were added.

2.2. *Plasma membrane preparation*

All procedures were at 4°C unless otherwise stated and buffers were made up as K^+ salts. The membrane preparation was modified from [8]. Log phase cells ($2–5 \times 10^6$ cells/ml) were washed twice in distilled water and resuspended in lysis medium (5 mM glycine, 0.2 mM PMSF, pH 8.5) at $2–5 \times 10^7$ cells/ml. After swelling for 5 min at room temperature the cells were lysed by 2 filtrations through a Millipore prefilter and a Whatman No. 3 filter. The lysate was cooled to 4°C and centrifuged at 7500 × *g* for 30 min. The crude membranes constituting the white top of the pellet were collected, resuspended in 50 mM glycine (pH 8.5) and the equivalent of $1–3 \times 10^9$ cells layered onto a sucrose step gradient (25%, w/w and 35%, w/w sucrose in 50 mM glycine, 0.2 mM PMSF, pH 8.5). After centrifugation at 120000 × *g* for 2½ h the interface material was collected with a pipette, and washed twice by centrifugation at 120000 × *g* for 1 h, first with 50 mM glycine, pH 8.5, and then with 50 mM glycine, 2 mM EDTA, pH 8.5. The membranes were then resuspended in ME buffer (10 mM Tris–HCl, 1 mM EDTA, 1 mM ATP, pH 7.5) at a concentration of >5 mg/ml and frozen at –70°C. In these conditions the ATPase was stable for over 3 months. Membranes at a protein concentration of 2 mg/ml were solubilized with 10 mg/ml lysolethicin in ME buffer for 10 min. After centrifugation at 100000 × *g* for 1 h the supernatant was layered on top of a continuous 6–20% (v/w) sucrose gradient in ME buffer, and centrifuged for 20 h at 120000 × *g*.

2.3. *Enzyme assays and analytical techniques*

Liposomes for the proton pumping assay were made by sonicating an asolectin suspension at 50 mg/ml in 10 mM MES, 25 mM K_2SO4, pH 6.7, in a Kerry KS100 bath sonicator until clarity. 100 μl of liposomes were mixed with protein from the sucrose gradient, K_2SO4 added to 25 mM and the mixture stored frozen in liquid nitrogen. 5–100 μl of thawed proteoliposomes were mixed with 1.34 ml of 10 mM MES, 25 mM K_2SO4, pH 6.7, ACMA at 0.03 μg/ml and the valinomycin to 1.5 μg/ml. Fluorescence was measured with a Perkin Elmer LS3 Fluorospectrometer connected to a chart recorder at an excitation wavelength of 400 nm and emission at the suboptimal wavelength of 525 nm. The initial fluorescence was taken as 100%.

Alkaline phosphatase was assayed as in [9], succinate dehydrogenase as in [10,11] and acid phosphatase as in [10,12]. ATPase was assayed at 30°C in 100 μl of 25 mM MES, 8 mM ATP, 16 mM MgCl, pH 6.8, plus 1–15 μg of protein.

After 5–60 min the reaction was stopped with 300 μl 7% SDS and the liberated inorganic phosphate measured by adding 1 ml of a solution that consisted of 1 part fresh 10% ascorbic acid plus 6 parts of 1 N sulphuric acid, 0.42% molybdate. After 20 min the absorbance at 720 nm was read.

Vanadate- and azide-sensitive ATPase were defined as the difference between the activity in the normal assay and an assay in the presence of 100 μM sodium vanadate or 5 mM azide respectively. This concentration of vanadate inhibited the ATPase activity in washed membranes by 91%.

Surface iodination with ^{125}I (Amersham, carrier free) was adapted from [13]. Protein was measured according to [14] with BSA as standard.

3. RESULTS

3.1. *Preparation and solubilisation of a plasma membrane ATPase*

For reasons of convenience, in the experiments described here, the plasma membrane proton pump was assayed as a vanadate-sensitive ATPase

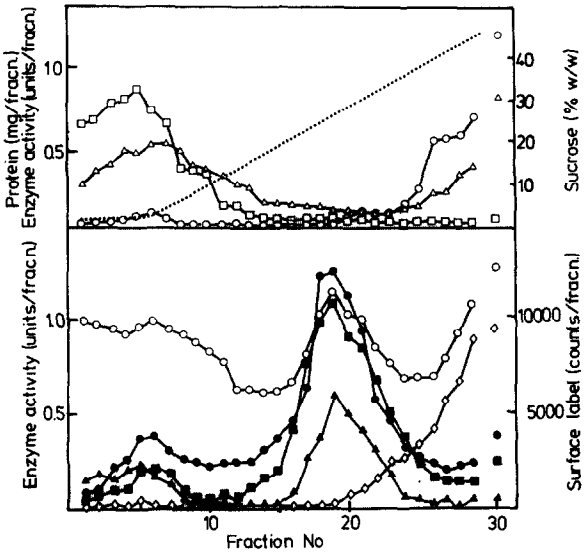

Fig. 1. Plasma membrane location of the vanadate-sensitive ATPase. Crude membranes (7500×*g* white pellet) were centrifuged through a sucrose density gradient as described in section 2, except that the step gradient was replaced by a continuous 5–45% w/w sucrose gradient. 1 ml fractions were collected and assayed. The vanadate-sensitive ATPase co-migrates with two plasma membrane markers, alkaline phosphatase and ^{125}I surface labelling and is separated from a mitochondrial marker, succinate dehydrogenase and a lysosomal marker, acid phosphatase. Top: ···, sucrose concentration; △, protein fraction; □, acid phosphatase (μmol/h); ○, succinate dehydrogenase (μmol/min). Bottom: ■, Vanadate-sensitive ATPase (μmol/min); ◇, azide-sensitive ATPase (μmol/min); ○, total ATPase (μmol/min); ▲, alkaline phosphatase (μmol/h); ● ^{125}I surface label. (Fraction 30 is the pellet material at the bottom of the gradient.)

[3,4]. The first step was to prepare purified plasma membranes with a high vanadate-sensitive ATPase activity. This was done using a modified version of [8], which involves lysis of the cells by forced filtration followed by differential and isopycnic centrifugation. Other methods were less satisfactory [15–17]. Electron microscopy of the washed membranes showed smooth membrane vesicles similar to those shown in other plasma membrane preparations [18]. Recognisable cells, nuclei or mitochondria were completely absent (not shown). The morphology of the membrane preparation and

Table 1

Distribution of enzyme markers during the plasma membrane preparation

Fraction	Total protein (mg)	Vanadate-sensitive ATPase (μM/mg per min)	^{125}I surface label (cpm/mg)	Alkaline phosphatase (μM/mg per h)	Succinate dehydrogenase (μM/mg per h)	Azide-sensitive ATPase (μM/mg per min)	Acid phosphatase (μM/mg per min)	Total ATPase (μM/mg per min)
1. Homogenate	2860	0.04	100	0.14	0.08	0.1	0.17	0.8
2. Crude membranes	331	0.17	407	0.4	0.27	0.25	0.36	0.63
3. 25–35% interface	11.2	1.0	1360	1.4	0.42	0.15	0.02	2.1
4. Washed membranes	7.0	1.6	1630	1.1	0.27	0.1	0.03	1.9
5. Solubilized membranes	4.5	2.9	1980	1.6	0.16	0.09	0.015	3.2

There is nominally a 72-fold purification of the vanadate-sensitive ATPase in the solubilized membranes, compared with a 20- and 11-fold purification of the membrane markers ^{125}I and alkaline phosphatase. The ATPase and markers become separated in steps 3–5

the copurification of the vanadate-sensitive ATPase with two markers for the plasma membrane, alkaline phosphatase and ^{125}I surface label, strongly suggest that this ATPase is located in the plasma membrane (fig. 1 and table 1).

Trial detergent solubilisations of the washed membranes were performed at a constant protein concentration of 0.25 mg/ml, and the ATPase was considered solubilized if it did not pellet after centrifugation at 100000 × *g* for 1 h. The only detergent that solubilized without loss of activity was the phospholipid lysolecithin. The following detergents solubilized at least 50% of the protein at the indicated concentration: Triton X-100, 1 mg/ml; cholate 8 mg/ml; octylglucoside 16 mg/ml; Zwittergent Z10 10 mg/ml; Z12 2 mg/ml; Z14 0.8 mg/ml; Z16 1 mg/ml; but the ATPase activity was greatly reduced, usually to 0–20%. The detergents CHAPS, CHAPSO and Zwittergent Z8 solubilized less than 10% of the protein but still inactivated the ATPase. In some cases it was possible to reactivate the ATPase by assaying it in the presence of added lysolethicin. In order to remove excess lysolethicin (which interferes with the proton pumping assay), the solubilized material was centrifuged through a sucrose gradient. Both the vanadate-sensitive ATPase and the proton pumping activity formed a broad peak centering at about 9% sucrose (see section 2).

3.2. *Demonstration of an electrogenic proton pump*

The solubilized ATPase from the sucrose gradient was reconstituted into asolectin liposomes by freeze-thawing [19]. Proton pumping was detected using the self-quenching fluorescent dye, ACMA. This is a weak base and therefore equilibrates across the liposome membrane according to the proton concentration gradient, so that high, self-quenching concentrations occur within the liposomes, when the inside becomes sufficiently acid. Fig. 2 shows that there is slow quenching of fluorescence when the reconstituted pump is incubated with Mg-ATP. The rate of quenching increased sharply when the K^+-specific ionophore valinomycin was added to dissipate charge gradients, from which we conclude that the pumping is electrogenic. The proton-specific ionophore CCCP released quenching, confirming that quenching was due to the existence of a proton concentration gradient.

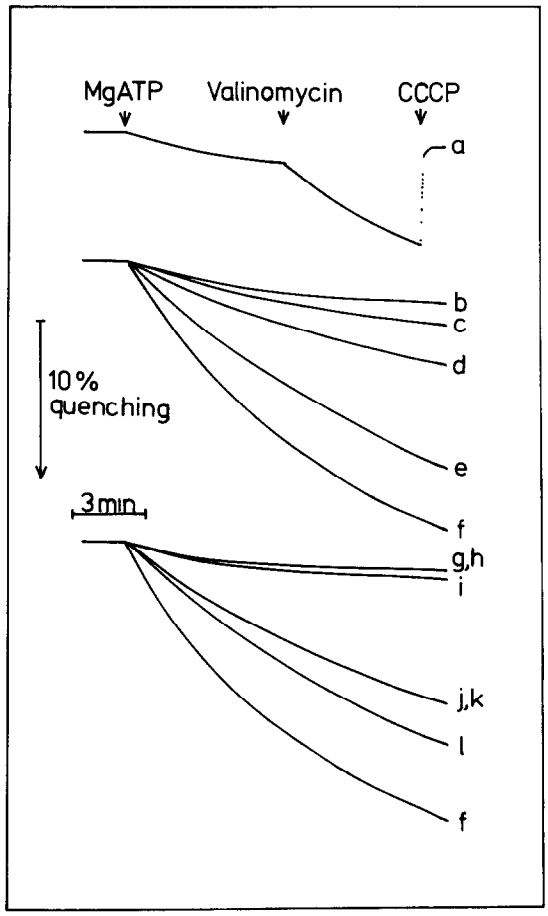

Fig. 2. Properties of the reconstituted proton pump. In this assay quenching of the fluorescence indicates acidification of the interior of the liposomes. Traces are redrawn so that the initial linear parts superimpose and they are corrected for the quenching that occurs immediately after the addition of MgATP; this quenching is not due to enzymatic activity. Trace (f) shows the quenching with proteoliposomes (10 μg/ml protein, 600 μg/ml asolectin), 4 mM Mg-ATP, valinomycin 1.5 μg/ml and variations from these conditions are as follows: (a) the addition of valinomycin was delayed and the final quenching was released by CCCP. The ATP concentration was reduced to 0.1 mM (b) or the ATP replaced by 4 mM CTP (c) or UTP (e). In (d) the proteoliposome concentration was reduced to 3.3 μg/ml protein and the asolectin to 200 μg/ml. Inhibitors were added as follows: miconazole 30 μM (g), 6 μM (j); DES 40 μM (h), 12 μM (l), vanadate 150 μM (i), 20 μM (k).

Table 2

Substrate specificities of the membrane bound ATPase and the reconstituted proton pump

Substrate	Phosphate release assay		Proton pumping assay	
	K_m (mM)	SA (μmol/min per mg)	K_m (mM)	SA (% quenching/ min per mg)
Na-ATP	nd	0.12	nd	<10
Mg-ATP	0.9	1.9	$0.5<K_m<1.5$	810
Mg-GTP	1.2	1.4	nd	nd*
Mg-UTP	1.8	0.7	$1.5<K_m<5.0$	233
Mg-CTP	2.3	0.5	$1.5<K_m<5.0$	93

*As both GTP preparations available interfered with the pumping assay by reducing quenching no values were calculated. nd = not determined. Specific activities were measured with 8 mM substrate and the pumping assay was done with 11 μg/protein and 2.2 mg/ml asolectin. A maximum of 50% quenching was achieved. To release this quenching an acidification of the medium of 3.5 pH units was required, strongly suggesting that the proton pump can generate a pH gradient of this magnitude

3.3. *Kinetic properties of the Mg^{2+}-ATP-dependent proton pump*

Kinetic parameters were measured using the ATPase activity in washed membranes and key points confirmed using the more qualitative proton pumping assay (table 2). The solubilized ATPase was stable between pH 4.5 and 9.0, and had a broad pH optimum at around pH 6.8 with less than 50% of the activity below pH 5.7 and above 8.0.

Varying the Mg^{2+} to ATP ratio showed that Mg-ATP is the true substrate of the enzyme [20]. Other nucleotides could be utilized to lesser degrees, when activity was measured both by phosphate production or by proton pumping (table 2 and fig. 2). The specific proton pump inhibitors vanadate, DES and miconazole inhibited the ATPase activity in washed membranes by 50% at 5 μM, 25 μM and 30 μM, respectively. These inhibitors were less effective at high lipid concentrations, so that vanadate halved the rates of ATP hydrolysis and pumping at 28 μM and 22 μM, respectively, in the presence of 600 μg/ml asolectin, the concentration which is used in the pumping assay. The lipid-soluble DES and miconazole also reduced quenching (fig. 2), but the values may not be a precise measure of proton pumping as control experiments showed that both substances made liposomes leaky. The reaction product ADP inhibited both the ATPase and the pumping competitively (not shown).

The divalent cations Hg^{2+} and Cu^{2+} were effective inhibitors in both assays with I_{50} values of 5 and 130 μM, respectively. Other divalent cations were much less effective inhibitors in the sequence of $Zn^{2+} > Co^{2+} > Ca^{2+} > Fe^{2+} > Mn^{2+}$. Inhibition by Ca^{2+} was not potentiated by bovine calmodulin (<10% inhibition at 50 μM Ca^{2+} and calmodulin at 0.2 μg/μg protein). Cyclic AMP, which stimulates a proton efflux from intact cells [21] gave no measurable stimulation of ATPase and proton pumping activity at concentrations up to 100 μM cAMP. A mixture of Na^+ and K^+, each at 100 mM, stimulated activity by only 10% and this was most likely an ionic strength effect. The absence of a significant Na^+/K^+ ATPase activity was confirmed by the lack of inhibition by ouabain (only 7% inhibition at 300 μM). Azide, even at 5 mM, inhibits ATP-hydrolysis and proton pumping by 5% or less (see table 1).

As the results presented here show that there was a good correspondence between the ATPase and the proton pumping activities in all parameters measured, we conclude that both assays probably

reflect the same enzyme activity. It appears that the proton pump is the major ATPase activity in our plasma membrane preparation.

4. DISCUSSION

There is no doubt that the *Dictyostelium* proton pumping ATPase is not the mitochondrial ATPase due to its location, its lower pH optimum and its insensitivity to mitochondrial ATPase inhibitors. As we had no specific markers for vacuoles, and there are reports that alkaline phosphatase can be associated with vacuoles [22,23], we cannot rule out the possibility that the ATPase is vacuolar in origin and co-purifies with plasma membranes. However this seems unlikely as vacuolar proton pumps [24–26] are insensitive to vanadate, have a low K_m for ATP of about 0.1–0.2 mM and are stimulated by Cl^-. On the other hand, the properties of the *Dictyostelium* ATPase are very similar to the proton pumps isolated from plasma membranes of *Neurospora* [4], yeast [3,27] and plants [28]. Not only does it share the same apparent location, but it also has a similar high K_m for ATP of 0.9 mM and is sensitive to the same range of inhibitors. It differs from the yeast proton pump in accepting other nucleotides besides ATP as substrates, but is similar to the *Neurospora* ATPase in this respect (see [29]). Although it has been suggested that the hydrolysis of other nucleotides might be due to contaminating mitochondrial ATPase [5] this seems unlikely in our case as their hydrolysis was resistant to azide and sensitive to vanadate. Earlier workers investigating the ATPase activity of plasma membranes in *Dictyostelium* [18,30] were attempting to detect a mammalian-type Na^+/K^+ ATPase and we agree with their conclusions that such an enzyme probably does not exist in growing *D. discoideum* cells.

The pH optimum of the pump is close to the intracellular pH and because membranes from cells adapted to growth at pH 5.0 possess about 3-fold more pump activity than those from cells grown at pH 6.5 (unpublished), it is attractive to suppose that this proton pump is involved in regulating intracellular pH. We plan to investigate the inhibitory effects of DIF and a number of non-physiological stalk cell inducing agents on the pump.

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