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*.

**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 (pHᵢ) regulates this choice, with low pHᵢ favouring stalk cell differentiation.

In other free living eukaryotes such as yeast and fungi and also in plants, pHᵢ 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that DES and miconazole can induce stalk cell differentiation, mimicking the action of the natural inducer DIF ([1] and unpublished).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 pHᵢ 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.

**2. MATERIALS AND METHODS**

Asolectin (phosphatidylcholine) was from Associated Concentrates, NY.

ACMA was a generous gift from R. Kraayenhof.
(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.

2.3. 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 K₂SO₄, pH 6.7, in a Kerry KS100 bath sonicator until clarity. 100 μl of liposomes were mixed with protein from the sucrose gradient, K₂SO₄ 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 K₂S₀₄, 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%.

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 × 10⁶ 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 × 10⁷ 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 × 10⁹ cells layered onto a sucrose step gradient (250–70, 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 lysolecithin 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.

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.
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

![Graph](image)

Table 1

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

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 125I 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 I251 surface label, strongly suggest that this ATPase is located in the plasma membrane (fig. 1 and table 1).

Trial detergent solubilizations 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 100,000 × 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 lysolecithin. In order to remove excess lysolecithin (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

![Fig. 2. Properties of the reconstituted proton pump.](image-url)}
Table 2
Substrate specificities of the membrane bound ATPase and the reconstituted proton pump

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Phosphate release assay</th>
<th>Proton pumping assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_{\text{in}}$ (mM)</td>
<td>SA (μmol/min per mg)</td>
</tr>
<tr>
<td>Na-ATP</td>
<td>nd</td>
<td>0.12</td>
</tr>
<tr>
<td>Mg-ATP</td>
<td>0.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Mg-GTP</td>
<td>1.2</td>
<td>1.4</td>
</tr>
<tr>
<td>Mg-UTP</td>
<td>1.8</td>
<td>0.7</td>
</tr>
<tr>
<td>Mg-CTP</td>
<td>2.3</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*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 around pH 6.8 with less than 50% of the activity below pH 5.7 and above 8.0.

Varying the Mg$^{2+}$/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.

REFERENCES
