An H⁺-ATPase regulates cytoplasmic pH in *Pneumocystis carinii* trophozoites

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Pneumocystis carinii is an opportunistic fungus which causes interstitial pneumonia in patients with acquired immunodeficiency syndrome (AIDS). Cytoplasmic pH (pH_i) regulation in short-term-cultured *P. carinii* trophozoites was studied using the fluorescent dye 2',7'-bis-(2-carboxyethyl)-5-(-6)-carboxyfluorescein. With an extracellular pH of 7.4, the mean baseline pH_i of *P. carinii* trophozoites was 7.40 ± 0.10 (*n* = 8). This steady-state pH_i was not significantly affected in the absence of extracellular Na⁺ or K⁺. Moreover, steady-state pH_i was maintained in the nominal absence of HCO₃⁻ and was not affected by the Cl⁻/HCO₃⁻-exchanger inhibitor 4,4'-di-isothiocyanatodihydrostilbene-2,2'-disulphonic acid (100 μM), or the Na⁺/H⁺-exchanger inhibitor *N*-ethyl-*N*-isopropylamiloride (100 μM). In contrast, the general inhibitors of ATPases, *N*-ethylmaleimide (1 mM), and dicyclohexylcarbodi-imide (100 μM), and the in-

hibitor of yeast H⁺-ATPase, diethylstilbestrol (12.5–100 μM), decreased pH_i, while the K⁺/H⁺-ATPase inhibitor omeprazole (50–400 μM), and the vacuolar-type H⁺-ATPase inhibitor bafilomycin A₁ (1–5 μM) only produced a dose-dependent acidification of the cells when used at high concentrations. In addition, steady-state pH_i depended on the availability of cellular ATP, since it was decreased by the ATP synthase inhibitors oligomycin (1 μg/ml) and sodium azide (1 mM), and by the uncoupler of oxidative phosphorylation carbonyl cyanide *p*-trifluorophenylhydrazide (1 μM), agents that were able to deplete significantly the intracellular ATP levels. Taken together, these results are consistent with an important role of an H⁺-ATPase similar to those found in other fungi in the regulation of pH_i homeostasis in *P. carinii* trophozoites.

INTRODUCTION

Maintenance of cytoplasmic pH (pH_i) within the physiological range is crucial to normal cell function due to the narrow pH optima of many intracellular processes [1–6]. In most eukaryotic cells control of pH_i is accomplished by ion-coupled extrusion mechanisms of H⁺ equivalents. There are at least two families of transporters involved in the regulation of pH_i in most eukaryotic cells: (1) Na⁺/H⁺ exchangers, which electroneutrally raise pH_i, and can be inhibited by amiloride and its derivatives [2–4]; and (2) Na⁺-dependent and -independent HCO₃⁻ transporters, which alkalize or acidify the cytosol respectively, and can be inhibited by stilbene disulphonate derivatives [2–5]. In addition to these transporters, another pH_i regulatory mechanism exists in some eukaryotic cells [7–12]. ATP-dependent proton extrusion is known to occur in both normal cells [7–11] and mammalian tumour cells [12], as well as in micro-organisms [13,14]. In these cases, proton pumping is thought to be mediated by ATPases. These pumps occur in two forms, an electrogenic H⁺-ATPase, and an electroneutral H⁺/K⁺-ATPase. The plasma membrane H⁺-ATPase of micro-organisms such as fungi is of the so-called P-type and is inhibited by micromolar concentrations of diethylstilbestrol [13,14], while that present in mammalian cells [7–12] is of the V- or vacuolar-type, and is inhibited specifically by low concentrations of bafilomycin A₁ [15]. On the other hand, the H⁺/K⁺-ATPase, which is also of the P-type, is present mainly in the gastric mucosa, is involved in acid secretion, and is inhibited by low concentrations of omeprazole and related compounds [16].

Pneumocystis carinii is an opportunistic pathogen that has been recognized as the leading cause of morbidity and mortality in AIDS patients. However, very little is known about its life cycle and metabolic pathways [17]. At present, nothing is known about the regulation of pH_i homeostasis in this organism. However, pH_i regulation is essential for growth and differentiation of fungi. In *Candida albicans*, for example, a rise in pH_i precedes transformation from yeast to mycelial form [18], and a plasma membrane H⁺-ATPase has been implicated in this process. A gene encoding a P-type cation-translocating ATPase has been cloned from a genomic library of rat-derived *P. carinii* which is 69–75% identical with eight proton pumps from six fungal species and it has been postulated that this pump might be involved in the regulation of pH_i, although its plasma membrane location has not been investigated [19]. On the other hand, it has been shown that the Na⁺/H⁺-exchanger inhibitors amiloride and dimethyl amiloride reduced growth of *P. carinii* as compared with the untreated control in 7-day-old cultures [20].

Taking into account all these considerations, we investigated the mechanisms involved in pH_i homeostasis in *P. carinii* using intact 2',7'-bis-(2-carboxyethyl)-5-(-6)-carboxyfluorescein (BCECF)-loaded trophozoites.

MATERIALS AND METHODS

Short-term cultivation of *P. carinii*

Human embryonic lung fibroblasts (HEL-299, A.T.C.C. CCL137) were grown on Cytodex beads in spinner flasks

Abbreviations used: AM, acetoxymethyl ester; BCECF, 2',7'-bis-(2-carboxyethyl)-5-(-6)-carboxyfluorescein; H₂-DIDS, 4,4'-di-isothiocyanatodihydrostilbene-2,2'-disulphonic acid; DCCD, dicyclohexylcarbodi-imide; EIPA, *N*-ethyl-*N*-isopropylamiloride hydrochloride; FCCP, carbonyl cyanide *p*-trifluorophenylhydrazide; MEM, minimal essential medium; pH_i, cytoplasmic pH; pH_o, extracellular pH.

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containing minimum essential medium (MEM) and 10% (v/v) fetal calf serum, as described before [21], and inoculated with *P. carinii* obtained from infected rat lungs at a concentration of 7×10^5 trophozoites/ml. Cultures were incubated with stirring for up to 7 days at 37 °C. *P. carinii* was obtained from the culture as described previously [21].

The inocula were prepared by homogenizing *P. carinii*-infected rat lung in MEM, centrifuging the homogenates at 250 g to sediment large pieces of tissue, counting the numbers of trophozoites in 10 μ l Giemsa-stained samples of supernatant, and adjusting the volume of the supernatant appropriately to achieve the desired concentration of trophozoites per millilitre.

Chemicals and solutions

MEM, fetal calf serum, dicyclohexylcarbodi-imide (DCCD), diethylstilbestrol, *N*-ethylmaleimide, oligomycin, adenosine-5'-triphosphate reagents and NaN_3 were purchased from Sigma Chemical Co. Bafilomycin A_1 was from Kamiya Biomedicals (Thousand Oaks, CA, U.S.A.). Omeprazole was a gift from Astra Hässle AB (Mölnådal, Sweden) through the courtesy of Dr. K. Andersson. BCECF acetoxymethyl ester (BCECF/AM), 4,4'-di-isothiocyanatodihydrostilbene-2,2'-disulphonic acid (H_2 -DIDS) and *N*-ethyl-*N*-isopropylamiloride hydrochloride (EIPA) were from Molecular Probes (Eugene, OR, U.S.A.). All other reagents were of analytical grade.

The resuspension medium (standard buffer) contained 135 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 1 mM CaCl_2 , 10 mM Hepes/Tris, pH 7.4, and 5 mM glucose; $-\text{Na}^+$ medium was prepared by isosmotic replacement of NaCl by choline chloride; $-\text{K}^+$ medium was prepared by isosmotic replacement of KCl by choline chloride.

Intracellular pH measurements

Intracellular pH was measured fluorometrically using BCECF. After harvesting the cells, they were washed twice at 2000 g for 10 min at 4 °C in buffer A which contained 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO_4 , 5.5 mM D-glucose and 50 mM Hepes at pH 7.4. Cells were resuspended to a final density of 1×10^9 cells/ml in loading buffer which consisted of buffer A plus 1.5% (w/v) sucrose, and 6 μM BCECF-AM. The suspensions were incubated for 30 min in a 30 °C water bath with mild agitation. Subsequently, the cells were washed twice with the ice-cold resuspension buffer indicated in the Figure legends to remove extracellular dye. Cells were resuspended to a final density of 1×10^9 cells/ml in the same buffer and were kept on ice. For pH_i measurements, a 60 μl aliquot of the cell suspension was diluted into 3.0 ml of the buffer used (5×10^7 cells/ml final density) in a cuvette placed in a thermostatically controlled (30 °C) Hitachi F-2000 spectrofluorimeter. The fluorescence ratios, with wavelengths for excitation set at 505/440 nm and for emission at 530 nm were recorded and translated into pH values on the basis of the ratios obtained at various pHs [22]. Other experimental conditions and calibrations were as described before [22,23].

Acid loading was accomplished by adding 10 mM sodium or potassium propionate to 1.5×10^7 cells suspended in 3 ml of incubation medium [1].

ATP measurements

The cells were washed twice with buffer A as described above, and then resuspended in buffer A (5×10^7 cells in a total volume of 1 ml), and incubated for 5 min at 37 °C in the presence or absence of 1 $\mu\text{g}/\text{ml}$ oligomycin, 1 mM NaN_3 , or 1 μM carbonyl

cyanide *p*-trifluorophenylhydrazone (FCCP). At the end of the incubation period the cells were centrifuged and 1.0 ml of ice-cold 1.0 M HClO_4 was added to each cell pellet containing 5×10^7 cells. After 30 min incubation on ice, the extracts were centrifuged and the supernatants were neutralized by the addition of 1.0 ml of 0.72 M KOH/0.6 M KHCO_3 . The precipitated KClO_4 was removed by centrifugation, and the supernatant was kept on ice until use. ATP was measured by the reaction between 3-phosphoglycerate and ATP catalysed by phosphoglycerate kinase coupled to the oxidation of NADH by glyceraldehyde-3-phosphate dehydrogenase, as described by Adams [24].

RESULTS AND DISCUSSION

Steady-state pH_i

In the nominal absence of bicarbonate and with an extracellular pH (pH_o) of 7.4, the mean baseline pH_i of *P. carinii* trophozoites was 7.40 ± 0.1 (eight determinations from two different preparations) (Figure 1A). This steady-state pH_i level was maintained in either Na^+ -free (Figure 1B) or K^+ -free (Figure 1C) medium, thus indicating that neither ion was essential for maintaining basal pH_i under these conditions.

Characterization of HCO_3^- -independent pH_i recovery

To investigate further the role of Na^+ and K^+ in pH_i regulation, trophozoites loaded with BCECF were acid-loaded by addition of propionic acid, which readily enters the cytoplasm but is excluded from acidic compartments [5]. Addition of 10 mM sodium propionate to trophozoites suspended in standard buffer at pH_o 7.4, resulted in a 0.2 unit decrease in pH_i , as illustrated in Figure 2(A). When the same experiment was repeated in Na^+ -free (Figure 2B) or K^+ -free (Figure 2C) medium, acid-loaded cells recovered their initial pH_i at the same rate and up to the same level as cells suspended in standard buffer (Figure 2A). Taken together, these results (Figures 1 and 2) rule out the involvement of an Na^+/H^+ antiporter or a K^+/H^+ -ATPase in the maintenance of basal pH_i or in the recovery of the cells from an acid load. In agreement with these results, neither basal pH_i nor recovery from an acid load was different from control cells in

Figure 1 Steady-state pH_i of *P. carinii* trophozoites

The cells (1.5×10^8 cells/60 μl of buffer A) were centrifuged in an Eppendorf centrifuge at 2000 g for 1 min, and the pellet resuspended in 3 ml of standard buffer (control), sodium-free buffer ($-\text{Na}^+$) or potassium-free buffer ($-\text{K}^+$). Other experimental conditions are described in the Materials and methods section. Traces are representative of two independent experiments conducted on separate cell preparations.

Figure 2 Effect of medium composition on HCO_3^- -independent pH_i recovery of acid-loaded trophozoites

The cells were suspended in (A) standard buffer, (B) sodium-free buffer ($-\text{Na}^+$) or (C) potassium-free buffer ($-\text{K}^+$). Where indicated by the arrows 10 mM sodium (A, C) or potassium (B) propionate (PROP) was added. Other conditions were as in Figure 1. Traces are representative of three independent experiments conducted on separate cell preparations.

the presence of up to 100 μM EIPA (an inhibitor of the Na^+/H^+ exchanger [25]) or up to 25 μM omeprazole (an inhibitor of gastric H^+/K^+ -ATPase [16]) (results not shown). However, omeprazole caused a dose-dependent decrease in basal pH_i when used at higher concentrations (50–400 μM , results not shown). In this regard, it has been demonstrated that when used at high concentrations omeprazole can inhibit other H^+ -ATPases [16].

Effect of inhibitors on steady-state pH_i

Addition of 100 μM H_2 -DIDS to *P. carinii* trophozoites did not cause any cytoplasmic acidification (Figure 3C). Since steady-state pH_i was maintained in the nominal absence of HCO_3^- (Figure 1), was not inhibited by H_2 -DIDS (Figure 3C), and was independent of the presence of Na^+ in the extracellular medium (Figure 1B), these results indicate that neither an Na^+ -dependent nor an Na^+ -independent HCO_3^- transporter is involved in its regulation, and suggest that the main mechanism for maintenance of pH_i and for recovery from an acid load in trophozoites could involve an ATP-dependent H^+ extrusion mechanism.

Two classes of proton-translocating ATPases have been described in the plasma membrane of eukaryotic cells that could be involved in pH_i regulation: (1) the P-type, such as the H^+/K^+ -ATPase present in the luminal membrane of gastric epithelial

Figure 3 Effect of inhibitors on steady-state pH_i of trophozoites

The cells were treated as in Figure 1, and resuspended in standard buffer. Where indicated one of the following inhibitors was added: 1 μM FCCP (B), 100 μM H_2 -DIDS (C), 100 μM *N*-ethylmaleimide (NEM, D), 1 mM NaN_3 (E), 100 μM DCCD (F), 1 $\mu\text{g}/\text{ml}$ oligomycin (OLIGO, G), 25 μM diethylstilbestrol (DES, H), and 5 μM bafilomycin A_1 (BAF, I). Control was without inhibitors present (A). Other conditions were as in Figure 1. Traces are representative of two to four independent experiments conducted on separate cell preparations.

cells [16] (the presence of which was ruled out by the results shown above) or the H^+ -ATPase present in the plasma membrane of yeast [13], and (2) the vacuolar-type H^+ -ATPase, present in macrophages [5], osteoclasts [7], hepatocytes [9], epithelial cells [10], secretory cells from the urinary tract [8] and intercalating cells of the renal collecting duct [11], as well as in several tumour cells [9,12]. Bafilomycin A_1 is a very specific inhibitor of vacuolar-type H^+ -ATPases when used at low concentrations [15]. Basal pH_i was not significantly affected by addition of up to 0.1 μM bafilomycin A_1 (results not shown), although higher concentrations of this compound (5 μM) caused a decrease in pH_i (Figure 3I, trace b). However, pH_i returned to control levels (Figure 3I, trace a) after 400 s (Figure 3I, trace b), suggesting that the decrease in pH_i was caused by inhibition of the vacuolar H^+ -ATPase of acidic intracellular compartments and recovery pro-

Figure 4 Effect of different concentrations of diethylstilbestrol on the steady-state pH_i of trophozoites

The cells were treated as in Figure 1, and resuspended in standard buffer. The Figure shows the pH_i measured after 10 min incubation in the presence or absence of different concentrations of diethylstilbestrol. Values shown are the mean $\pm$ S.D. of duplicates from three representative experiments.

ceeded by a bafilomycin A₁-insensitive mechanism. Vanadate, a known inhibitor of P-type ATPases, had no effect on basal pH_i up to a concentration of 0.5 mM (results not shown). However, vanadate is charged and it does not penetrate easily into intact cells. In fact, in studies with intact cells vanadate has been shown to mimic the effects of hormones such as insulin [26], and the demonstration of intracellular effects required membrane permeabilization [27]. In contrast, basal pH_i was decreased by addition of the general H⁺-ATPase inhibitors DCCD (100 μM, Figure 3F) and *N*-ethylmaleimide (1 mM, Figure 3D). The yeast H⁺-ATPase inhibitor diethylstilbestrol [13] caused a decrease in basal pH_i (Figure 3H), which was dose-dependent with maximal activity at 50 μM (Figure 4). In neither case was recovery to the original steady-state pH_i observed. Furthermore, addition of either 1 μg/ml oligomycin (Figure 3G) or 1 mM NaN₃ (Figure 3E) (inhibitors of the mitochondrial ATP-synthase) or 1 μM FCCP (an uncoupler of mitochondrial oxidative phosphorylation, Figure 3B) greatly decreased pH_i. In this regard, after incubation of the parasites for 5 min at 37 °C in the presence of 1 μg/ml oligomycin, 1 mM NaN₃ or 1 μM FCCP, their ATP levels dropped significantly to $58 \pm 3.9\%$, $53 \pm 1.6\%$ and $53 \pm 1.7\%$, respectively, of the control values (0.072 ± 0.016 μmol of ATP/10⁸ cells). Taken together, these results suggest the

requirement of ATP for maintaining basal pH_i. It would be interesting to know if the product of the gene that has been cloned from a genomic library of rat-derived *P. carinii* [19], and which has homology with other fungal P-type cation-translocating ATPases, is located in the plasma membrane and corresponds to the proton pump that we found to be involved in pH_i regulation in this organism.

In summary, regulation of pH_i in *P. carinii* appears to occur by mechanisms different from those present in mammalian cells. Our results support the hypothesis that fungal plasma membrane proton pumps could be promising targets for chemotherapy of opportunistic infections caused by these micro-organisms [14].

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REFERENCES

- Swallow, C. J., Grinstein, S. and Rotstein, O. D. (1988) *J. Biol. Chem.* **263**, 19558–19563
- Aronson, P. S. (1985) *Annu. Rev. Physiol.* **47**, 545–560
- Roos, A. and Boron, W. F. (1981) *Physiol. Rev.* **61**, 296–434
- Boron, W. F. (1983) *J. Membr. Biol.* **72**, 1–16
- Swallow, C. J., Grinstein, S. and Rotstein, O. D. (1990) *J. Biol. Chem.* **265**, 7645–7654
- Cabantchik, Z. I., Knauf, P. A. and Rothstein, A. (1978) *Biochim. Biophys. Acta* **515**, 239–302
- Väänänen, H. K., Karhukorpi, E.-K., Sundquist, B., Wallmark, B., Roinen, I., Hentunen, T., Tuukkanen, J. and Lakkakorpi, P. (1990) *J. Cell Biol.* **111**, 1305–1311
- Van Adelsberg, J. and Al-Awqati, Q. (1986) *J. Cell Biol.* **102**, 1638–1645
- Stone, D. K., Xie, X.-S., Wu, L.-T. and Racker, E. (1984) in *Hydrogen Ion Transport in Epithelia* (Forte, J. and Rector, F. C., eds.), pp. 219–230, Wiley, New York
- Turrini, F., Sabolic, I., Zimolo, Z., Moewes, B. and Burckhardt, G. (1989) *J. Membr. Biol.* **107**, 1–12
- Schuster, V. L., Fejes-Toth, G., Naray-Fejes-Toth, A. and Gluck, S. (1991) *Am. J. Physiol.* **260** (Renal Fluid Electrolyte Physiol. **29**), F506–F517
- Martinez-Zaguilan, R., Lynch, R. M., Martinez, G. M. and Gillies, R. J. (1993) *Am. J. Physiol.* **265**, C1015–C1029
- Serrano, R. (1988) *Biochim. Biophys. Acta* **947**, 1–28
- Monk, B. C. and Perlin, D. S. (1994) *Crit. Rev. Microbiol.* **20**, 209–223
- Bowman, E. J., Siebers, A. and Altendorf, K. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7972–7976
- Mattsson, J. P., Väänänen, K., Wallmark, B. and Lorentzon, P. (1991) *Biochim. Biophys. Acta* **1065**, 261–268
- Cushion, M. T., Harmsen, A., Matsumoto, Y., Stringer, J. R., Wakefield, A. E. and Yamada, M. (1994) *J. Med. Vet. Mycol.* **32**, 217–228
- Monk, B. C., Niimi, M. and Shepherd, M. G. (1993) *J. Bacteriol.* **175**, 5566–5574
- Meade, J. C. and Stringer, J. R. (1995) *J. Euk. Microbiol.* **42**, 298–307
- Shaw, M. M., Kleyman, T. R., Bartlett, M. S. and Smith, J. W. (1995) *J. Euk. Microbiol.* **41**, 110S
- Lee, C.-H., Bauer, N. L., Shaw, M. M., Durkin, M. M., Bartlett, M. S., Queener, S. F. and Smith, J. W. (1993) *J. Clin. Microbiol.* **31**, 1659–1662
- Negulescu, P. A. and Machen, T. E. (1990) *Methods Enzymol.* **192**, 38–81
- Docampo, R., Scott, D. A., Vercesi, A. E. and Moreno, S. N. J. (1995) *Biochem. J.* **310**, 1005–1012
- Adams, H. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 539–543, Academic Press, New York
- Kleyman, T. R. and Cragoe, E. J. (1988) *J. Membr. Biol.* **105**, 1–21
- Tamura, S., Brown, T. A., Whipple, J. H., Fujita-Yamaguchi, Y., Dubler, R. E., Cheng, K. and Kraft, A. S. (1984) *J. Biol. Chem.* **259**, 6650–6658
- Trudel, S., Downey, G. R., Grinstein, S. and Paquet, M. R. (1990) *Biochem. J.* **269**, 127–131