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Short Communication

Inhibition of H^+ Extrusion by Phosphocreatine in *Candida albicans*

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Vanadate, a potent inhibitor of P-type ATPases, reduces the electrochemical gradient considerably. H^+-extrusion in cells of *Candida albicans*, a pathogenic yeast, was strongly inhibited in the presence of 25 mM phosphocreatine (PCr) by about 83%. H^+-extrusion was further inhibited by 25 mM PCr in the presence of vanadate; 89% with 1 mM, 92% with 2 mM and 99% with 5 mM vanadate. 2 mM vanadate caused 90%, 92% and 96% inhibition in the presence of 20 mM, 30 mM and 40 mM PCr, respectively. Creatine (Cr) had a negligible effect on H^+- extrusion. The inhibition caused by 1 mM, 2 mM and 5 mM vanadate alone was 66%, 77% and 88%, respectively. PCr and vanadate inhibit proton extrusion with almost equal magnitude. It can be concluded that phosphate moiety of PCr interacts with the ATPase and is similar to vanadate interaction. Since PCr is having such a drastic inhibitory effect on ATPase activity we can say that it is playing a significant role in holding a check on this pathogenic fungus in healthy human hosts.

Key words: *Candida albicans*, H^+-extrusion, vanadate, phosphocreatine, creatine.

Candida albicans is a dimorphic yeast and is believed to be an obligate associate of warm-blooded animals. It is usually present as a harmless asymptomatic commensal but can manifest as a pathogen. The pathogenecity of this opportunistic fungus is due to its capacity to induce germ tube formation (1). The cell membrane of yeast possesses an H^+-ATPase that nurtures intracellular pH and generates an electrochemical gradient of protons necessary for secondary transport systems. It uses the free energy for ATP splitting to translocate protons from the cell interior to the medium. The development of PM-ATPase as a molecular target for antifungal drug therapy (2) requires the demonstration that inhibition of enzyme activity correlates with cessation of cell growth.

Phosphocreatine (PCr) is the sole phosphagen in vertebrates. It has been shown to influence ATP dependent enzymes in invertebrate species and is found in association with virtually all types of ATPases. It serves as an energy carrier connecting sites of energy production with sites of energy utilization with the sub-cellularly compartmentalised creatine kinase (CK) isoenzymes. The effect of PCr on the rate of H^+-extrusion, pH_i and dimorphism in *C. albicans* has already been investigated (3) and it has been observed that physiological concentrations of PCr inhibit H^+-extrusion and delay dimorphism. It even alters pH_i pattern of cells destined to differentiate but the site of binding and mode of interaction is still not clear. Vanadate, a potent inhibitor of the H^+-ATPase (4,5) inhibits by competing with phosphate moiety of ATP for binding sites in the enzyme since they are analogous in structure. In the present communication, mode of interaction of PCr with H^+-ATPase has been studied and correlated with effects of vanadate.

All biochemicals and enzymes were obtained from Sigma Chemical Company, USA and all inorganic chemicals from Merck (India). Stock cultures of *Candida albicans* (ATCC 10261) were maintained on nutrient agar. To initiate growth for experimental purposes, cells from an agar culture were inoculated into yeast extract-peptone-dextrose (YEPD) medium (High Media) pH 6.8 and were grown at 30 °C up to stationary phase. The stock cultures were maintained on nutrient agar slants at 4 °C. To initiate growth for experimental purposes, cells from an agar culture were inoculated into a nutrient medium YEPD (Yeast Extract 1%, Peptone 2% and Dextrose 2%) and were grown at 30 °C for 24 h i.e., up to stationary phase (primary culture). The cells were re-inoculated into a fresh YEPD medium and grown for 8-10 h i.e., up to mid-log phase (secondary culture).

Mid-log phase cells harvested from YEPD medium were washed twice with double distilled water and routinely 200 mg cells were suspended in 10 ml solution containing 0.1 M KCl and 0.1 mM $CaCl_2$ in all the experiments including control. Suspension was kept in a double-jacketed glass

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Abbreviations: PCr, phosphocreatine; Cr, creatine; CK, creatine kinase.

container with constant stirring. The container was connected to a water circulator at 25 °C. pH was monitored using a pH-meter for 30 min. Proton extrusion rate was calculated from the volume of 20 mM NaOH consumed in automatic titration in pH-stat mode of Autotitrimeter (Radiometer ETS 822, Copenhagen) over a period of 10 min. Increments and rate of delivery of titrant was adjusted according to demands of the experiments and were routinely 100 ml and 40 ml min^{-1}. Initial pH was adjusted to 7.0 using 0.01 M HCl/NaOH. PCr, Cr and vanadate were added to the cell suspension after adjusting the pH to 7.0 and then recordings were noted after every minute.

H^+-extrusion by *Candida* cells in the presence of different concentrations (1 mM, 2 mM and 5 mM) of vanadate and 25 mM PCr is shown in Fig. 1. As expected the cells of untreated control showed acidification starting from pH 7.0. However, the acidification decreased in the presence of vanadate; the decrease being more with higher concentration (5 mM) of vanadate. Similarly 25 mM PCr also showed decrease in acidification, the magnitude being very similar to 5 mM vanadate. Fig. 2 shows the effect of different concentrations of PCr on H^+-extrusion in the presence of 2 mM vanadate. The PCr concentrations were taken within the physiological range (20-40 mM). It is clearly indicated that in the presence of all PCr concentrations the H^+-extrusion decreased tremendously. The acidification almost changed to alkalination. The inhibition of H^+-extrusion increased with increasing concentrations of

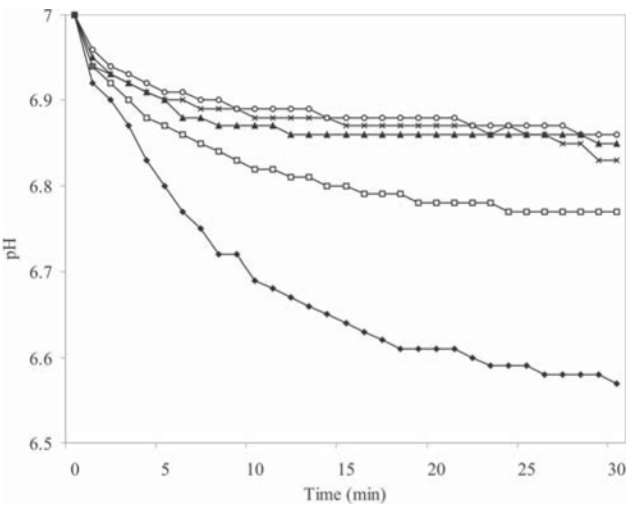

Fig. 1. Effect of different concentrations of vanadate and PCr on H^+-extrusion by *Candida albicans*. *Candida* cells were present in 0.1 mM $CaCl_2$ and 100 mM KCl. (–◆– control; –□– 1 mM Vanadate; –×– 2 mM Vanadate; –○– 5 mM Vandate; –▲– 25 mM PCr)

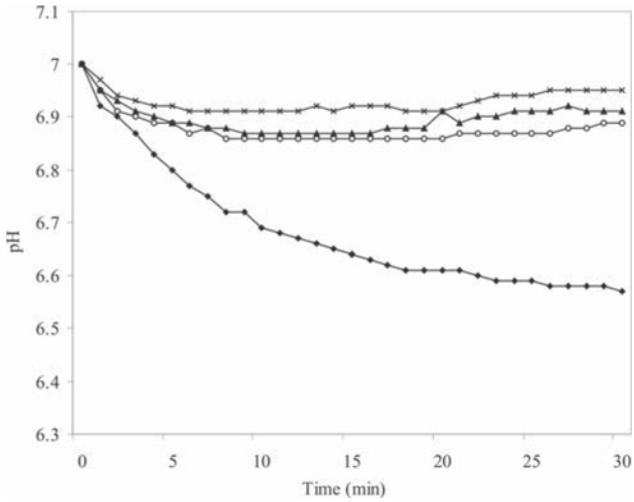

Fig. 2. Effect of different concentrations of PCr on H^+-extrusion by *Candida albicans* in the presence of 2 mM Vanadate. *Candida* cells were present in 0.1 mM $CaCl_2$ and 100 mM KCl. (–◆– control; –○– 20 mM PCr; –▲–30 mM PCr; –×– 40 mM PCr)

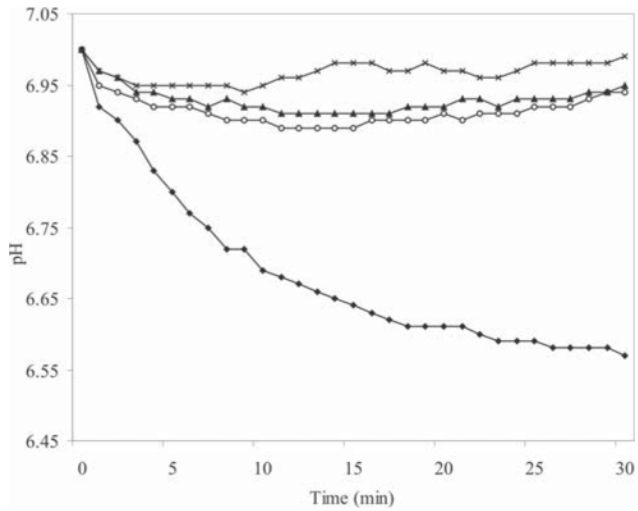

Fig. 3. Effect of different concentrations of Vanadate on H^+-extrusion by *Candida albicans* in the presence of 25 mM PCr. *Candida* cells were present in 0.1 mM $CaCl_2$ and 100 mM KCl. (–◆– control; –○– 1 mM Vanadate; –▲– 2 mM Vanadate; –×– 5 mM Vandate)

PCr. The results presented in Fig. 3 show the effect of different concentrations of vanadate (1 mM, 2 mM and 5 mM) on H^+-extrusion in the presence of 25 mM PCr. Here also the effect was inhibitory and showed a trend towards alkalination; inhibition being greater for higher concentration of vanadate.

Table 1 gives the effect of various PCr concentrations on the rate of H^+-extrusion by *Candida* cells in the absence and presence of different concentrations of vanadate at

Table 1. Effect of various concentrations of PCr on the rate of H^+-extrusion by *Candida* cells in the absence and presence of different concentrations of vanadate at pH 7.0 and 25 °C

Incubation mixture	H^+-extrusion rate (x 10^{-11} mol min^{-1} mg^{-1} cells)	% Inhibition
Cells only (control)	3.55	–
Cells + 1 mM Vanadate	1.20	66
Cells + 2 mM Vanadate	0.80	77
Cells + 5 mM Vanadate	0.40	88
Cells + 25 mM PCr	0.61	83
Cells + 2 mM Vanadate + 20 mM PCr	0.35	90
Cells + 2 mM Vanadate + 30 mM PCr	0.30	92
Cells + 2 mM Vanadate + 40 mM PCr	0.15	96
Cells + 25 mM PCr + 1 mM Vanadate	0.40	89
Cells + 25 mM PCr + 2 mM Vanadate	0.30	92
Cells + 25 mM PCr + 5 mM Vanadate	0.03	99

pH 7.0 and 25 °C. 25 mM PCr showed 89%, 92% and 99% inhibition of H^+-extrusion in the presence of 1 mM, 2 mM and 5 mM vanadate, while 25 mM PCr alone showed 83% inhibition. This shows that vanadate which is an established inhibitor of this ATPase (6) is inhibiting further. 2 mM vanadate showed 90%, 92% and 96% inhibition in the presence of 20 mM, 30 mM and 40 mM PCr, while 1 mM and 5 mM vanadate alone showed only 66% and 88% inhibition.

The rate of H^+-extrusion in yeast cells was also studied for various concentrations of creatine. Control showed an extrusion rate of 3.12 x 10^{-11} mol min^{-1} mg^{-1} cells. The average effect on extrusion rate brought about by various Cr concentrations was however, insignificant (results not shown).

From these studies we can see that both PCr and vanadate inhibit the proton extrusion with almost equal magnitude which indicate that both PCr and vanadate bind to the ATPase and bring conformational changes almost in the same manner. Cr alone has no effect on H^+-extrusion. Comparing the structure of PCr and Cr it seems that inhibition occurs due to the "phosphate" moiety and not the "Cr" moiety of PCr. The structure of vanadate, a potent inhibitor of PM-ATPase, is analogous to the structure of phosphate [$VO_4^{3-} \cong PO_4^{3-}$]. It may thus be binding to the site where ATP binds *via* its phosphate. PCr and vanadate both have produced a cumulative effect when the cells were exposed to both these compounds together. Both of them may be having more than one binding sites. These studies further confirm the findings that PCr does interact with the H^+-ATPase inhibiting it to a great extent.

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