Biochimica et Biophysica Acta, 972 (1988) 277–282
Elsevier

BBA 12369

Dimorphism-associated changes in intracellular pH of *Candida albicans*

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(Received 23 February 1988)
(Revised manuscript received 28 June 1988)

Key words: Cell divergence; pH, intracellular; (*C. albicans*)

Intracellular pH (pH_i) was monitored during pH-regulated dimorphism of *Candida albicans* using two different methods: (1) by steady-state distribution of propionic acid and (2) by use of polyene antibiotic, nystatin. There was no significant change in pH_i during the first 120 min in either bud- or germ tube-forming populations. However, there was a rapid increase around 135 min which also coincided with the time of evagination. The magnitude of increase in pH_i was different in the two populations; being 0.44 and 0.14 pH units in bud- and germ tube-forming populations, respectively. In the two diverging populations, the transient increase in pH_i was followed by a rapid drop. The sharp rise in pH_i of the population destined to form buds was sensitive to orthovanadate and to the depletion of K^+ from the medium while this was not the case with germ tube-forming cells. The results suggest that pH_i may play an important role in the phenotypic divergence of *C. albicans*.

Introduction

There is growing evidence that cytoplasmic pH (pH_i) may have an important role in mitogenic signal pathways. Growth-factor stimulation of quiescent fibroblast cells, in particular, has been shown to induce a rapid intracellular alkalinization which has been linked to amiloride sensitive Na^+/H^+ antiporter activity [1–6]. Evidence that pH_i is indeed involved in the control of cell proliferation comes from several sources [1,2]. Recently, the transformation of quiescent fibroblast cells by microinjection of activated *ras* gene product has also been shown to be preceded by a transient cytoplasmic alkalinization [7]. In addition, changes in intracellular pH affect cell shape [8], morphological transitions [9], cytoskeleton assembly [10], and aggregation and secretion [11] in a variety of cells.

Candida albicans is a dimorphic and pathogenic yeast. Its dimorphism is of special relevance, since it is an important factor in the pathogenesis of candidosis [12]. It also provides an unusually simple model for investigating the mechanisms which regulate cellular differentiation [13]. Apart from the mandatory temperature, morphological transition can be induced by a host of factors [14–21]. The pH-regulated dimorphism is especially interesting because it can easily be regulated with minimum variation in growth conditions. In view of the importance of pH_i in growth and differentiation of mammalian cells [3,10,11] and in phenotypic divergence of *Candida albicans* [12,22,23], the present work was planned to assess the importance of intracellular pH in dimorphism. The results of this communication demonstrate that a transient rise in intracellular pH precedes pheno-

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typic divergence of *C. albicans,* and the magnitude of change in pH_i was greater in the evaginating population destined to diverge as buds when compared to that of the germ tube-forming population. These findings suggest that pH_i may be a contributory agent in the differentiation of *C. albicans.*

Materials and Methods

Organisms and growth conditions

C. albicans ATCC 10261 was obtained from Dr. M.G. Shepherd, New Zealand. Cultures were maintained on agar slants at 4° C [24]. These cells were inoculated into 25 ml of amino acid-rich medium (pH 6.8) [24] in plastic Erlenmeyer flasks, and were grown at 25° C upto the late log phase of growth ($5 \cdot 10^7$ cells per ml). For zinc depletion [22], cells were inoculated into a fresh flask and maintained at 25° C at stationary phase for 48 h, resulting in a final concentration of $2.5 \cdot 10^8$ cells per ml.

Induction of differentiation

Stationary phase G_1 singlets ($1.5 \cdot 10^9$ cells) maintained as described earlier were lightly sonicated at a power of 5 μm peak to peak amplitude, using a MSE Ultrasonic Disintegrator at 2–4° C for 1 min and incubated into 300 ml nutrient media in 500 ml Erlenmeyer flasks [25]. Cells were grown at 37° C at either pH 4.5 or 6.5 to induce bud or hypha formation, respectively [12,13,22, 24–26]. Aliquots were removed at different time

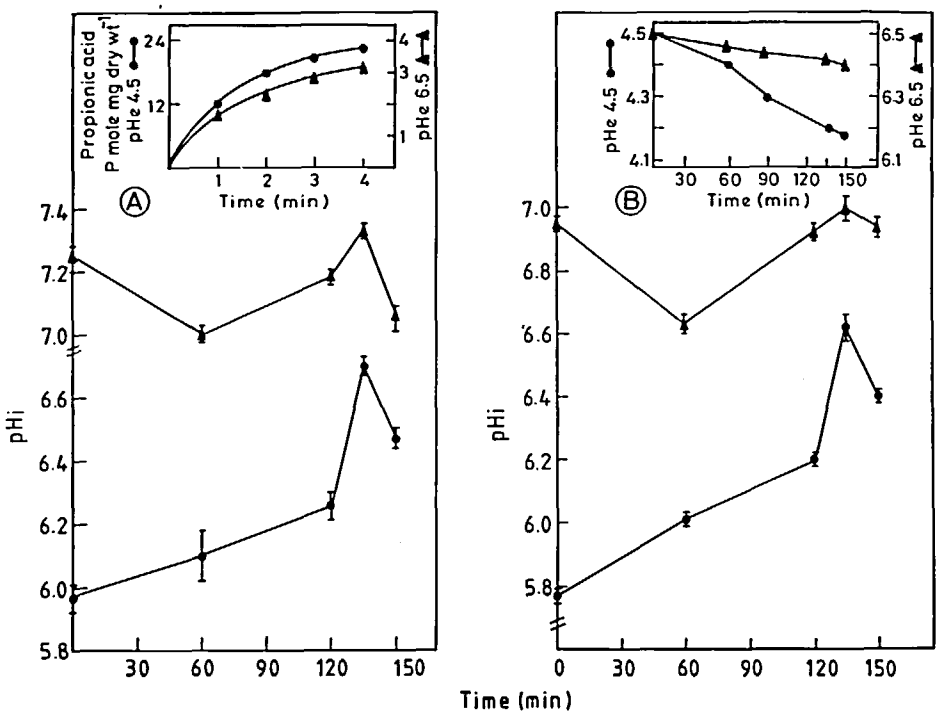

Fig. 1. (A) Intracellular pH (pH_i) as a function of time during pH_e regulated dimorphism in *C. albicans.* Cells, as described in Materials and Methods, were induced to differentiate to bud phenotype at pH 4.5 (●) and to hyphal phenotype at pH 6.5 (▲). The pH_i was measured as described earlier [28], using [^{14}C]propionate. Each point is mean of 3–4 determinations, and bars represent the ±S.E. values. Inset (A) shows the time-course of steady-state distribution of [^{14}C]propionic acid in cells at pH 4.5 (●) and 6.5 (▲), respectively. (B) Intracellular pH (pH_i) as a function of time during pH_e-regulated dimorphism. Details of the experiments were similar to those described for Fig. 1A. Cells were allowed to differentiate to bud phenotype at pH 4.5 (●) and hyphal phenotype at pH 6.5 (▲). The pH_i was measured using Nystatin (10 μM). Each point is mean of 3–4 determinations and bars represent the ±S.E. values. Inset (B) shows pH_i as a function of time when pH of the growth medium was not adjusted at pH 4.5 (●) and 6.5 (▲). (Details as described in Materials and Methods).

intervals, and cell divergence was microscopically monitored. The external pH of the medium was checked using a Beckman pHI 71 pH meter and pH was adjusted every half hour to the original pH with microdroplets of NaOH or HCl (Fig. 1B inset). Cells were washed twice with double-distilled water before being used for further experiments.

Measurement of intracellular pH

Internal pH (pH_i) values were calculated by determining the steady-state distribution of propionic acid across the plasma membrane [27]. Assay conditions were similar to those described elsewhere [28]. A reaction mixture containing 100 mM Tris-citrate buffer (pH 4.5 or 6.5) and yeast cells (10–15 mg dry wt per ml) was incubated at 30 °C. The distribution of propionic acid was initiated by the addition of sodium [2-^{14}C]propionate (10 μM, 18.5 kBq/ml). It was earlier established that 3 min were required for propionic acid to equilibrate across the plasma membrane (Fig. 1A inset). For actual pH_i determinations, triplicate samples (1 ml) were removed after 3 min and rapidly filtered through Maxflow filter discs (0.45 μm). Cells retained on the filters were washed four times with 1 ml of ice cold, 100 μM, buffer (pH 4.5 or 6.5) containing 10 μM unlabelled propionic acid. Radioactivity retained was counted in a Beckman LS 1801 Beta liquid scintillation counter using a toluene-based scintillation fluid. pH_i values were calculated as described earlier [27,28].

In another method, the polyene antibiotic, nystatin, was used to measure pH_i [29]. This method has been shown to give reliable pH_i values closer to the true intracellular pH and comparable to other indirect methods of pH_i measurement. Nystatin (10 μM, dissolved in dimethyl formamide) was added to a 2.5% unbuffered aqueous cell suspension. Prior to the addition of the antibiotic, the initial external pH (pH_e) of the suspension was adjusted to the pH of its growth (i.e., pH 4.5 and 6.5) with very dilute Tris and citrate. The change in pH of the suspension was followed at 20 °C on a PHM 84 pH meter (Radiometer, Copenhagen, Denmark) with constant stirring. The value of external pH at which nystatin permeabilization induced no further shift in external pH was taken as an estimate of pH_i [29].

Chemicals

Sodium [2-^{14}C]propionate was purchased from Amersham-Buchler, Braunschweig, F.R.G. Sodium orthovanadate, propionic acid and nystatin were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. All other chemicals used were of analytical grade or of the highest purity available commercially.

Results and Discussion

The synchronized stationary phase G_1 singlets, grown as described in Materials and Methods, were incubated at two different external pH values (4.5 and 6.5) to differentiate as buds and hyphae, respectively. This method provided two exclusively diverging populations of either buds or of germ tubes [24]. Soll and his co-workers [25] have demonstrated that under such conditions the cells begin to evaginate around 135 min. The evagination coincides with the time of commitment of the cell to diverge as buds. However, reciprocal shift experiments have revealed that the time of commitment of cells to diverge as germ tubes is several minutes later [23]. Internal pH was monitored by using a steady-state distribution ratio of [^{14}C]propionic acid [27,28]. Our earlier results had shown that pH_i was maintained fairly constant between pH_e 3.5 and 6.5, however, it increased gradually with further increase in pH_e [28]. Fig. 1A and B depict the pH_i changes during the course of differentiation to either bud or germ tube form. It is important to mention here that the initial internal pH of the two populations was different because of the different pH_e values to which the cells were exposed [24]. The pH_i did not significantly change up to 120 min, but there was a rapid increase in intracellular pH around 135 min, which was followed by an equally rapid drop in both the populations. The magnitude of increment of pH_i in the two populations was, however, different being 0.44 pH units in buds and 0.14 pH units in germ tubes.

The exposure of unbuffered cell suspension to nystatin has also been used as a reproducible method for pH_i measurements [29,30]. Nystatin interacts with the sterols of plasma membrane to produce nonspecific pores which permit the equilibration of protons (and other ions) across the

membrane. Nystatin does not interact with mitochondria [29], thus this method provides pH_i values closer to the true cytoplasmic pH. By this method, the pattern and magnitude of pH_i changes were similar to those observed by propionate distribution (Fig. 1A), but their absolute values differed (Fig. 1B). Thus the pH_i measurements by two independent methods gave comparable results.

The movement of protons across the plasma membrane of many yeasts and fungi is controlled by a plasma membrane-bound H^+-translocating ATPase [31–35]. The electrochemical gradient of H^+ is responsible for most secondary transports [36–38]. The fluxes of H^+/K^+ (H^+/Na^+ in case of animal cells) also control pH_i [3,4,6,39]. Results, so far, suggest that, similar to other yeasts and fungi, *Candida albicans* also possesses a plasma membrane-bound ATPase [31,40]. Since we observed pH_i changes during phenotypic divergence, it was of interest to ascertain the role of H^+-translocating ATPase in the maintenance of pH_i. Orthovanadate, a potent inhibitor of plasma membrane bound H^+-ATPase [40], was added to both the populations (exposed to either pH_e) before the incubation was started. The increase in pH_i of evaginating cells was inhibited in a concentration-dependent manner by orthovanadate (data not shown). At 500 μM concentration of the inhibitor, the rise in intracellular pH observed in the 'control' set could be partially reversed in the budding population (Fig. 2A) but the pH_i changes in germ tube-forming population were insensitive. Most of the cells (90%) destined to form buds remained unbudded. The percentage of germ tube formation was not much affected; however, they were smaller in size. It should be pointed out that in the presence of orthovanadate (which at-

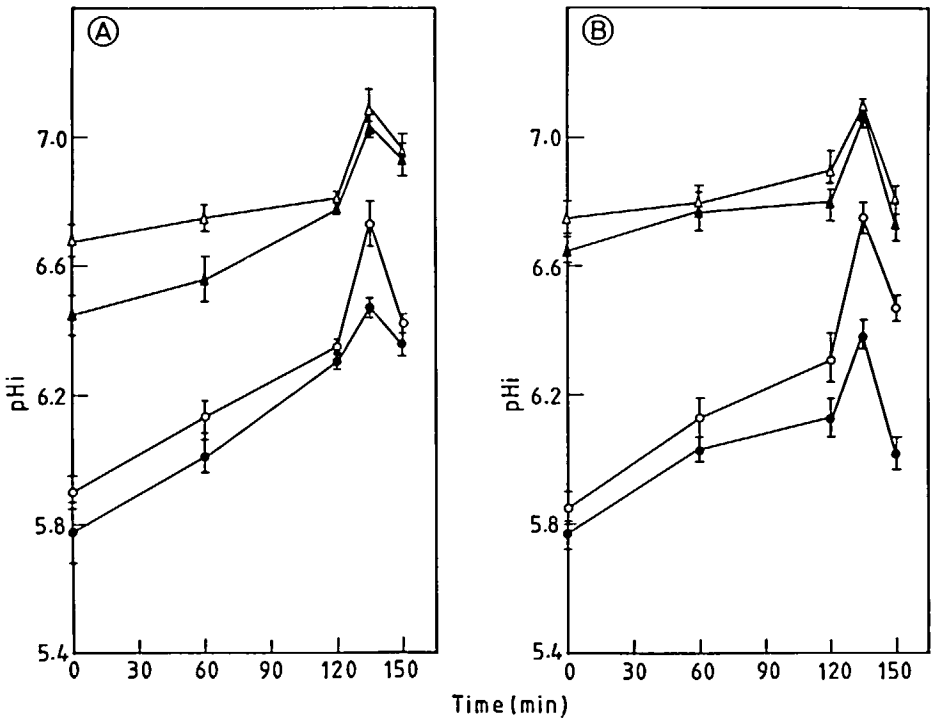

Fig. 2. (A) Effect of orthovanadate on pH_i during divergence of *C. albicans* (pH_i was measured using Nystatin). Stationary phase cells were allowed to diverge at pH 4.5 in the presence (●) or absence (○) of 500 μM orthovanadate, and at pH 6.5 in the presence (▲) or absence (△) of the same concentration of orthovanadate. Each point is the mean of 3–4 determinations, and bars represent the ±S.E. values ($P < 0.05$). (B) Role of K^+ on pH_i during divergence of *C. albicans*. Cells were induced to diverge in the presence (○) or absence (●) of K^+ at pH 4.5, and in the presence (△) or absence (▲) of K^+ at pH 6.5. pH_i was measured using Nystatin. Each point is the mean of 3–4 determinations, and bars represent the ±S.E. values.

tenuates pH_i rise), the population favouring bud formation did not form germ tubes instead, because a continuous exposure to high external pH (6.5) is essential for germ tube formation. Soll and his co-workers [25] were able to reverse the phenotypic divergence by shifting the population destined to form buds (grown at pH 4.5) to pH 6.5, and that destined to form germ tubes (grown at pH 6.5) to pH 4.5.

Dissipation of excessive membrane potential by cationic accumulation is essential for the maintenance of ionic equilibrium across the plasma membrane [41,42]. K^+ is known to be the compensatory counter-ion of H^+ that maintains electroneutrality across the plasma membrane in yeasts [42]. The incubating media, at pH 4.5 and 6.5, was depleted of K^+ (the potassium phosphate in Lee's modified media was replaced by an equimolar amount of sodium phosphate). This depletion prevented the sharp rise in pH_i of bud-forming cells; however, this was not the case with the germ tube-forming population. During the course of differentiation the cells destined to form buds appeared shrunken and did not evaginate, indicating that K^+ cannot be replaced by Na^+. Germ tube formation was, however, not significantly affected. Depletion of K^+ from the growth medium of *Streptococcus mutans* has also been shown to regulate shape transitions. Under such conditions there was no effect on the bacillary form, whereas the growth of the coccoid form was affected adversely [8].

The present data suggests that pH_i may play an important role in the differentiation of *C. albicans*. Results indicate that the rise in pH_i is concomitant with evagination during both bud and germ tube formation. In contrast to the rise in pH_i during germ tube formation, the transient rise in budding cells appears to be a necessary event. Furthermore, the rise in pH_i during the course of differentiation into bud phenotype seems to be regulated by the plasma membrane bound ATPase mediated by the K^+/H^+ antiporter [42]. Our results indicate that the preference of *C. albicans* towards a certain differentiation pathway is not only dependent on external pH [25], but fluctuations in pH_i may also play an equally important role. With respect to this, it is interesting to note that changes in pH_i of *Dictyostelium* at the onset of the developmental programme, led to significant changes in its preference towards terminal phenotypes [9].

Acknowledgements

P. Mishra gratefully acknowledges the SRF award from C.S.I.R., New Delhi, India. This work was supported in part by a grant from D.S.T. (No. 22(7P/22)84 STP II) and I.C.M.R. (No. 5/11 (1)/87-BMS-II (8700080)) New Delhi, India. The special assistance from U.G.C. under COSIST is also acknowledged.

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