Influence of Na⁺ and anions on the dimorphic transition of *Candida albicans*

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The effect of Na⁺ (Cl⁻ or gluconate salt) on growth and dimorphic potential of the pathogenic yeast *Candida albicans* has been examined. Profiles of germ tube formation as a function of salt addition, pH and temperature indicated Na⁺ inhibition of germ tube outgrowth at high ambient pH (pH 8.0) which was exacerbated by replacement of Cl⁻ with gluconate (as an impermeant analogue). At acidic pH (pH 5.5) and permissive temperature (37 °C), gluconate alone promoted the dimorphic transition. Rates of glucose-induced medium acidification and plasma membrane H⁺-ATPase activity have been measured to assess whether salt treatments could retard the cytoplasmic alkalization known to precede germ tube formation. The precise site of Na⁺ action remains unclear but the anion effects may be interpreted in terms of anion-exchanger and channel activity acting to modulate cytosolic pH.

Keywords: Na⁺, lithium, anions, *Candida albicans*, pH regulation

INTRODUCTION

The dimorphic transition between yeast and hyphal forms of the human pathogenic yeast *Candida albicans* can be triggered or restrained by a variety of exogenous factors, including ambient pH, nutritional status and temperature (Odds, 1985). The Na⁺ and Cl⁻ concentrations of the defined media used in *C. albicans* whole-cell studies vary in their resemblance to those of the fluids which bathe the cells of the mammalian host of *Candida*. For example, the Na⁺, K⁺ and Cl⁻ concentrations of Lee's medium (Na⁺:K⁺:Cl⁻ in mM, respectively, 86:28:86; Lee *et al.*, 1975) rather simulate that reported for vaginal fluid (Na⁺:K⁺:Cl⁻ in mM 61:23:62; Levin & Wagner, 1977) but differ greatly from the composition of tissue fluid (Na⁺:K⁺:Cl⁻ in mM 132:4:102; Levin & Wagner, 1977). In contrast, a medium such as Yeast Nitrogen Base (YNB) has little physiological relevance in terms of electrolyte composition (Na⁺:K⁺:Cl⁻ in mM 0.1:0.5:0.09; Difco technical data). To date, studies on germ tube induction have largely neglected any possible effect of Na⁺ or its balancing anion Cl⁻ despite the preponderance of this salt in the *in vivo* microenvironment of this yeast. As the propensity of the yeast form to switch to hyphal growth may be clinically relevant (the hyphal form may allow penetrative infection of internal organs), it is thus of

interest to examine the effects of Na⁺ and Cl⁻ on the dimorphic transition.

In this study, acidic and alkaline ambient pH and permissive/non-permissive temperature regimes have been used to initiate *C. albicans* germ tube formation in the presence of Na⁺ as a Cl⁻ or gluconate (as an impermeant Cl⁻ analogue) salt. The external proton concentration may itself act as a dimorphic trigger (e.g. Buffo *et al.*, 1984), which could be of relevance to the distribution and state of *C. albicans* in its human host, given pH variation within and between infection locations (e.g. alkaline saliva cf. variably acidic vulvo-vaginal fluid). Moreover, external pH may be relevant to Na⁺ tolerance in yeast where the plasma membrane (PM) Na⁺ extrusion mechanism is driven by the transmembrane H⁺ electrochemical potential gradient (generated by the PM H⁺-ATPase). The best characterized example to date in this respect is *Schizosaccharomyces pombe*, which employs such an Na⁺-H⁺ antiporter to expel cytoplasmic Na⁺ (Jia *et al.*, 1992). No antiporter homologues have been reported for *C. albicans*, nor has a homologue of the *Saccharomyces cerevisiae* PM Na⁺-extruding ATPase (which could render Na⁺ expulsion independent of the H⁺ gradient; Haro *et al.*, 1991) been identified. It is timely then to examine whether ambient Na⁺ and H⁺ concentrations together influence basic growth properties of *C. albicans*, as a precursor to more detailed transport studies.

Abbreviation: PM, plasma membrane.

Possible interactive effects of pH with Cl^- have also been addressed here to assess the possibility that this anion is involved in the regulation of *C. albicans* cytosolic pH (pH_i). Although reports place pH_i of the yeast form just below neutrality (Stewart *et al.*, 1988; Rabaste *et al.*, 1995), rapid and profound transient cytoplasmic alkalization is a well-established phenomenon which precedes germ tube emergence in *C. albicans* (e.g. Stewart *et al.*, 1988) and is thought to be mediated at least in part by increased activity of the PM H^+ -ATPase (Monk *et al.*, 1993). Such primary pumps do not act alone but rather in concert with other ion transport systems (channels and exchangers) in electrically coupled arrays which serve to regulate membrane voltage and internal ion concentration. For example, in yeast vacuoles a Cl^- channel may regulate membrane voltage and so permit profound luminal acidification by the vacuolar H^+ -ATPase (Wada *et al.*, 1992). In human platelet cell membranes, a H^+ -ATPase is functionally coupled to a HCO_3^- - Cl^- exchanger, which regulates pH_i (Valant & Haynes, 1995). Here, by examining the effects of external Cl^- and its impermeant transport analogue gluconate (Larsen & Rasmussen, 1982) on germ tube formation under induction conditions known to result in pH_i modulation, it may be possible to infer whether Cl^- transport systems are involved in pH homeostasis and dimorphic transition.

METHODS

Culture and growth conditions. *Candida albicans* (CAI-4 Ura⁻ auxotroph generated by Fonzi & Irwin, 1993) was maintained on Sabouraud Dextrose Agar at 23 °C. This strain was chosen to facilitate future molecular biological studies; preliminary experiments showed that salt responses of a clinical isolate were similar (J. Davies, unpublished data). Liquid cultures were initiated in YNB (with amino acids; Difco) supplemented with 1% (w/v) glucose and 25 µg uridine ml⁻¹ (pH 5.4). This medium was selected to satisfy nutritional requirements whilst providing an effectively negligible baseline for Na^+ , Cl^- and K^+ (in mM 0.1, 0.09 and 0.5, respectively; Difco technical data). Growth was at 27 °C, with 200 r.p.m. orbital shaking to stationary phase (48 h). These cells were used to inoculate experimental cultures to a final density of 1×10^6 ml⁻¹ (30 ml medium in 100 ml volume flasks). The control experimental liquid medium comprised YNB, 1% (w/v) glucose, 25 µg uridine ml⁻¹ and 20 mM Tris/MES (pH 5.5 or 8.0); test medium was supplemented with up to 200 mM salt prior to pH adjustment. In addition to Cl^- and gluconate salts of Na^+ , the K^+ salts were tested to determine monovalent cation specificity. To this end, LiCl and choline chloride were also tested. All reagents were analytical grade unless otherwise stated. As an osmotic control, D-sorbitol (98% purity; Sigma) was added to raise medium osmolarity to that measured for the 200 mM sodium gluconate test by freezing point depression osmometry (549 mosM; all salt test media osmolarities were measured as not more than 9% below this value). Cells were grown at 27 or 37 °C at 200 r.p.m. for up to 8 h, after which medium pH was not stable.

Growth and germ tube determinations. Growth was determined as increase in OD_{840} (1 cm light path cuvettes, Shimadzu OPI-2 spectrophotometer; Karley *et al.*, 1997) against a growth medium blank. The specific growth rate (μ)

for yeast cells at pH 5.5 and 27 °C was calculated as $\mu = (\ln \text{OD} - \ln \text{OD}_0)/t - t_0$ (Harvey, 1973), where OD_0 and OD were the optical densities at times t_0 (4 h) and t (6 h), respectively. Germ tube formation was examined using an improved Neubauer haemocytometer; the morphological criteria of Pollack & Hashimoto (1987) were adopted whereby a germ tube was defined as a projection of at least 2 µm, lacking a constriction at its base. Percentage formation of germ tubes was estimated by sampling approximately 80–100 cells. Such percentage values were then converted to arcsin values [where $\sin \theta = \sqrt{(\% / 100)}$; John & Quenouille, 1977], so that standard error (SE) values did not exceed the 100% level and are reported as such. Statistically significant differences in control versus test germ tube formation rates are indicated in the text.

Extractable ATP assay. The method of Cockayne & Odds (1984) was modified for extractable ATP determination. After 5 h growth at 27 °C in control or test medium (pH 5.5), 0.5 ml cell suspension was added to 0.5 ml extraction buffer (50 mM Tris, 2 mM EDTA, 50 mM MgSO_4 , pH 7.75, with acetic acid), pre-heated in a water bath. After 4 min, the solution was cooled on ice then centrifuged at 1000 g (4 °C) for 5 min. The ATP content of a 0.5 ml aliquot of supernatant was determined using a preparatory kit (Sigma Diagnostics) which employs the coupled phosphoglycerate phosphokinase/glyceraldehyde phosphate dehydrogenase reaction described by Adams (1963). The rate of NADH oxidation was followed at 340 nm using a 1 cm light-path cuvette (Shimadzu OPI-2 spectrophotometer).

Permeabilization of yeast cells. The method was adapted from Kaur & Mishra (1991). Cells were harvested by centrifugation and washed with 30 ml ice-cold deionized water. Approximately 0.9 g (fresh wt) cells were resuspended in 5 ml suspension medium (0.1 M KCl; 4 mM MgCl_2 ; 8 mM imidazole; 10%, w/v, sorbitol; pH 6.5). Yeast cells were permeabilized by addition of 0.5 ml permeabilizing mixture [toluene/ethanol/10% (v/v) *t*-octylphenoxy-polyethoxy-ethanol (1:4:1, by vol.)] and vigorous shaking for 5 min in a Mickel vibrating mixer. This was followed by immediate centrifugation and three washes with 30 ml ice-cold suspension medium. Cells were resuspended in 5 ml suspension medium and placed on ice.

ATPase and protein assay. The ATPase assay was adapted from Darley *et al.* (1995) and Kaur & Mishra (1991). Aliquots of permeabilized cells containing 20–25 µg protein were made up to 100 µl with deionized water. Cells were then added to 200 µl reaction mixture and incubated at 30 °C for 10 min. The control reaction mixture contained 50 mM KCl, 5 mM MgCl_2 , 20 mM MES and 5 mM MgATP, buffered to pH 6.7 with Tris. Inhibitors of PM ATPase (10 mM sodium orthovanadate), mitochondrial ATPase (1 mM sodium azide) and phosphatases (1 mM ammonium molybdate) were included as appropriate and KNO_3 replaced KCl for nitrate inhibition of tonoplast ATPase. The reaction was stopped by the addition of 900 µl Ames reagent (six parts 0.4%, w/v, ammonium molybdate in 0.5 M H_2SO_4 to one part 10%, w/v, ascorbic acid; Ames, 1966). After standing for 1 h at room temperature, the A_{820} was read against the appropriate assay blank. Enzyme controls were run concurrently. Protein was estimated using the Bio-Rad assay kit, with bovine γ -globulin as the standard.

Glucose-induced acid extrusion assay. The glucose-induced medium acidification assay was adapted from Serrano (1980), as described by Karley *et al.* (1997). Yeast cells were grown as described previously in either control experimental medium or that supplemented with 200 mM NaCl or KCl (pH 5.5). Cells

were harvested by filtration (0.8 µm pore diameter filter; Whatman) after 5 h growth at 27 °C, washed with 15–20 ml ice-cold 5 mM MES/PIPES/Tris buffer (pH 5.5) and re-suspended in a final assay volume of 10 ml to a density of approximately 10⁷ cells ml⁻¹. The standard assay medium comprised 10 mM Tris/MES (pH 4.5–8.0). Salts were added to a maximum 10 mM concentration prior to pH adjustment. Water-soluble inhibitors were prepared in assay buffer; water-insoluble inhibitors were incorporated in DMSO/assay buffer, such that the final assay volume of DMSO did not exceed 0.1% (v/v). Control experiments showed that this DMSO concentration had no effect on acidification. Inhibitors were added to the cells immediately before glucose addition. Acidification was initiated by addition of a 100 µl aliquot of 200 mM glucose stock (made up in assay medium; final concentration 2 mM) and the time-course was followed using a Corning 240 pH meter with combination electrode and a Rikadenki chart recorder. Calibration was by addition of 5 µmol H⁺ equivalents. Assays were performed at room temperature (23 °C).

RESULTS AND DISCUSSION

Concentration-dependence of Na⁺ salt effects on germ tube formation at alkaline pH and non-permissive temperature

The effects of increasing concentrations of NaCl and sodium gluconate up to 200 mM at pH 8.0 and 27 °C on *C. albicans* germ tube formation after 8 h are shown in Fig. 1. At 200 mM, in sodium gluconate the yeast form was consistently retained ($n = 5$), with only a mean ($\pm$ SE) 21 $\pm$ 11% germination in NaCl ($n = 8$). Cells exposed to sodium gluconate remained viable as indicated by their ability to exclude methylene blue and

Fig. 1. Effect of NaCl and sodium gluconate additions on germ tube formation at pH 8.0 and 27 °C. Germ tubes were counted after 8 h growth, sampling 80–100 cells per individual trial; percentage values are reported after arcsin transformation as mean $\pm$ SE. Salt additions were made (in mM) as increasing concentrations of NaCl (○; $n = 8$) or sodium gluconate (●; $n = 5$). In trials where Na⁺ was maintained at 200 mM throughout, gluconate was varied from 40 to 200 mM with Cl⁻ added reciprocally to a final balancing anion concentration of 200 mM (□; $n = 7$).

Fig. 2. Time-course of germ tube emergence at pH 8.0. Cells were grown at 27 °C (open symbols) or 37 °C (filled symbols) with no salt addition (○, ●), 200 mM NaCl (□, ■), 200 mM sodium gluconate (▲) or 100 mM NaCl/100 mM sodium gluconate (▼). For clarity the 200 mM sodium gluconate (27 °C) trial has been omitted; in this, cells retained the yeast form. Percentage germ tube formation was estimated as described in Methods. Results are reported as mean $\pm$ SE of three independent trials.

concentrate neutral red in vacuoles. Na⁺ was also kept constant at 200 mM while the concentration of Cl⁻ and gluconate was varied within a total anion concentration of 200 mM (Fig. 1). At equimolar Cl⁻ and gluconate (i.e. 200 mM Na⁺, 100 mM Cl⁻ and 100 mM gluconate) mean germ tube formation was 18 $\pm$ 1% ($n = 7$). As the gluconate fraction increased at the expense of Cl⁻ (at constant Na⁺) so germ tube formation was depressed, indicating that retardation or inhibition of germ tube formation was not solely due to Na⁺ but was exacerbated by gluconate. Equally, this demonstrated that inhibition was not a consequence of increasing ionic strength. The mean germ tube formation for the sorbitol osmotic control (equivalent to 200 mM sodium gluconate) was 49 $\pm$ 2% ($n = 3$), which was not significantly different (Student's *t*-test) from the null addition control value of 57 $\pm$ 5% ($n = 11$). Both 200 mM NaCl and sodium gluconate treatments were significantly different at the 99% confidence level to the sorbitol control. The relatively low control germ tube formation rates could be a consequence of the high (55 mM) glucose concentration (Pollack & Hashimoto, 1987).

Time-course of germ tube formation at alkaline pH

The inhibitory effects of Na⁺ and gluconate at high pH could simply be a temporal phenomenon which only appeared significant as a result of the experimental time-course. Hence, the kinetics of germ tube formation were examined as a function of salt addition and temperature (Fig. 2). At 27 °C and pH 8.0 under null addition or sorbitol control conditions, germ tubes emerged after 2 h. Addition of 200 mM NaCl increased the lag period

Fig. 3. Germ tube formation profiles as a function of salt treatment, pH and temperature. Germ tube formation was estimated after 8 h growth at pH 8.0/27 °C (black bar), pH 8.0/37 °C (white bar) or pH 5.5/37 °C (hatched bar). Salts were added at 200 mM; sorbitol was added to generate the same osmotic potential as 200 mM sodium gluconate. Results are reported as mean $\pm$ SE from a minimum of three independent trials. Columns: 1, control; 2, NaCl; 3, sodium gluconate; 4, KCl; 5, potassium gluconate; 6, LiCl; 7, choline chloride; 8, sorbitol.

prior to germ tube emergence and caused a plateau response well below the apparent control steady-state number of germ tubes. Maintaining Na^+ at 200 mM but replacing 100 mM Cl^- with 100 mM gluconate further increased the lag period and depressed the steady-state level. These results suggested that the phenomenon was not only a retardation of the dimorphic response but also an inhibition and that a component of the effect could be attributed reasonably to external anions.

Use of the permissive temperature of 37 °C at pH 8.0 decreased the lag phase to germ tube emergence for all test conditions (Fig. 2). However, cultures were checked only at hourly intervals and so fine resolution of salt effects on this stage under these conditions remains unknown. The same pattern of depression of steady-state levels was observed, with sodium gluconate more inhibitory than NaCl.

Variation in germ tube formation with pH and temperature

To test whether inhibition of germ tube formation by NaCl and sodium gluconate was Na^+ -specific and to examine further pH and temperature dependency, the response was compared to K^+ salts, choline chloride (as a KCl transport analogue) and LiCl (with Li^+ as an Na^+ transport analogue; Haro *et al.*, 1991) at pH 5.5 or 8.0, 27 or 37 °C. The results of exposure to 200 mM salt additions are presented in Fig. 3. Qualitatively, the germ tube formation profiles in the presence of 200 mM KCl,

choline chloride and sorbitol closely resembled the control in that germ tube formation was most pronounced at (pH/ °C) 8/37, less so at 8/27 and most restricted at 5.5/37. The obvious deviations from this trend were obtained in the presence of 200 mM NaCl, sodium gluconate, potassium gluconate and LiCl. An osmotic component to the response was deemed unlikely as subjecting the control versus sorbitol test to a two-way ANOVA analysis (for all pH and temperature combinations used) gave *P* and *F* values for control/sorbitol interaction as 0.942 and 0.005 (to three decimal places), respectively, indicating that control and osmotically stressed cells responded similarly.

Effect of Na^+ on germ tube formation profile

A comparison of the Na^+ , K^+ and choline chloride salts at pH 8.0/27 °C (Fig. 3) indicated that in the presence of Cl^- , Na^+ specifically perturbed the germ tube formation response relative to either the null addition or osmotic control (significant differences at the 99% confidence level; Student's *t*-test). At pH 8.0 and 27 °C, LiCl caused a retention of the yeast form (significantly different from control at the 99.9% level; Student's *t*-test) but did not affect viability as indicated by vital staining with methylene blue; the interpretation of this effect in terms of Na^+ transport homology cannot be decisive as Li^+ is known to perturb eukaryote signal transduction systems (e.g. Manji *et al.*, 1996). For gluconate salts, in contrast to sodium gluconate, potassium gluconate permitted germ tube formation ($41 \pm 2\%$, $n = 4$), tending to confirm the premise of a specific Na^+ -inhibitory component to the sodium gluconate response at pH 8.0 and 27 °C.

At pH 8.0, a 10 °C increase to the permissive temperature of 37 °C (Fig. 3) enhanced the response of control and all test treatments. Germ tube formation in 200 mM NaCl and sodium gluconate was to levels which were not significantly different to the null addition and osmotic controls but the pattern observed in time-course experiments (i.e. sodium gluconate more inhibitory than NaCl; Fig. 2) was retained. Of all salts tested, only 200 mM LiCl produced a significantly different result to the control (99% level; Student's *t*-test). Comparison of NaCl with KCl and choline chloride suggested that an Na^+ -specific effect was still operative under alkaline/permissive temperature conditions, albeit ameliorated relative to 27 °C.

Maintaining the permissive temperature but decreasing pH to 5.5 (Fig. 3) decreased the mean control response from $84 \pm 5\%$ ($n = 5$) to $23 \pm 2\%$ ($n = 5$), a result anticipated from previous studies on pH regulation. In contrast to the responses at pH 8.0 (27 or 37 °C), NaCl was not inhibitory and sodium gluconate produced a statistically significant increase (at the 95% confidence level) in germ tube formation over null addition and sorbitol controls. These Na^+ salt responses at pH 5.5 and 37 °C were matched almost exactly by their respective K^+ salts [mean % $\pm$ SE (n): NaCl, 16 ± 1 (8); KCl, 16 ± 5 (3); sodium gluconate, 43 ± 8 (5); potassium

gluconate, 42 ± 3 (6). This suggested that the mechanism for Na⁺ inhibition was not effective at low pH and that the nature of the anion is again important in determining the germ tube formation response.

Influence of permeant and impermeant anions on germ tube formation profile

At alkaline pH, a comparison of NaCl against sodium gluconate and KCl against potassium gluconate at either 27 or 37 °C showed a consistently proportional difference between the anions irrespective of the cation (Fig. 3), i.e. at one temperature, the Cl⁻ salt effected an approximately 20% increase in the germ tube index over the gluconate salt. At 27 °C (pH 8.0), mean % $\pm$ SE germ tube formation in 200 mM NaCl was 21 ± 11 ($n = 8$) cf. sodium gluconate, 0 ($n = 3$); KCl, 59 ± 8 ($n = 3$) cf. potassium gluconate, 41 ± 2 ($n = 4$). Mean germ tube formation was significantly different from the controls at the 99% level (Student's *t*-test) for both Na⁺ salts. The mean potassium gluconate value was significantly different at the 98% level; the KCl value was not significantly different. At 37 °C the proportional effect was retained despite increased absolute values: NaCl, 79 ± 5 ($n = 4$) cf. sodium gluconate, 62 ± 6 ($n = 4$); KCl, 90 ± 0 ($n = 3$) cf. potassium gluconate, 75 ± 6 ($n = 3$). The other striking effect of anions on germ tube formation (described in the previous section) was gluconate-induced enhancement of the dimorphic transition at low pH and permissive temperature, which was independent of the major cation (Fig. 3). Of all salts tested at pH 5.5 and 37 °C, only sodium gluconate and potassium gluconate induced a significant increase in mean germ tube formation (95% level and 99.9% level, respectively; Student's *t*-test).

That external Na⁺ and anions were linked with ambient pH at a phenomenological level to the dimorphic transition suggested that these moieties may be involved in modulating germ tube formation via regulation of cytoplasmic pH (pH_i). In *Candida*, there is convincing evidence for a profound and rapid elevation in pH_i as an initial and perhaps deterministic event in the yeast/hyphal transition evoked by high pH and permissive temperature (Stewart *et al.*, 1988). As initial diagnostics of the physiological effects of external anions and the potentially inhibitory effect of Na⁺, the effects of the salts on the following properties of the yeast form (grown under non-permissive conditions to ease interpretation of the system) have been examined: (1) yeast growth as a simple test of Na⁺ tolerance; (2) ATP levels to test for a substrate restriction of PM H⁺-ATPase activity which could retard cytosolic alkalization; (3) PM ATPase activity in a permeabilized cell assay; (4) glucose-induced medium acidification.

Effects of salts on yeast cell growth rates and ATP content

The effects of 200 mM salt additions on the time-course of yeast cell growth (measured as increase in optical density) at pH 5.5 and 27 °C were measured and μ values

Table 1. μ values and extractable ATP content of yeast cells

Cells were grown in control medium or with the addition of 200 mM salt at pH 5.5 and 27 °C. Sorbitol was added to generate the same osmolarity as the sodium gluconate test for an osmotic control. Growth was measured as increase in optical density; μ was calculated as described in Methods for the 4–6 h growth interval. The estimation of ATP content was performed after 5 h growth. Results are expressed as mean $\pm$ SE (number of independent trials).

Growth medium	μ	ATP (nmol per 10 ⁶ cells)
Control	0.12 ± 0.010 (8)	47 ± 3 (3)
Sorbitol	0.15 ± 0.003 (3)	51 ± 5 (3)
NaCl	0.14 ± 0.010 (3)	40 ± 7 (4)
Sodium gluconate	0.17 ± 0.010 (4)	53 ± 3 (3)
KCl	0.15 ± 0.000 (3)	43 ± 4 (3)
Potassium gluconate	0.14 ± 0.010 (5)	42 ± 13 (4)
LiCl	0.09 ± 0.010 (4)	21 ± 4 (3)

were calculated. These are summarized in Table 1. The mean μ for the sorbitol osmotic control was significantly different from the null addition control at the 95% confidence level (Student's *t*-test). However, for salt additions relative to sorbitol only LiCl had a significant effect on mean μ . It is concluded from these results that cells were physiologically competent in the presence of either Na⁺ or gluconate and that neither could reasonably be described as cytotoxic at the concentrations deployed at low pH. Preliminary trials have shown that the CAI-4 strain yeast form can grow in YNB supplemented with 0.8 M NaCl at pH 5.5, maintaining growth rates comparable to the control values (F. Northrop & J. Davies, unpublished data). The effects of Na⁺ and gluconate on germ tube formation must have been more subtle.

Mean values of the extractable ATP concentration from yeast cells after 5 h growth at pH 5.5/27 °C are also given in Table 1. All values were of the same order of magnitude to those reported for *C. albicans* in previous studies (Cockayne & Odds, 1984; Karley *et al.*, 1997). There was no statistically significant difference between null addition and osmotic controls. With the exception of LiCl, there were no significant differences between salt treatments and the controls. Moreover, there were no significant effects between Cl⁻ and gluconate with either Na⁺ or K⁺ as the cation. It is therefore concluded that the inhibitory effects of Na⁺ and gluconate were unlikely to have resulted from restriction of metabolic ATP supply to the PM H⁺-ATPase, nor was gluconate stimulation of germ tube formation at pH 5.5/37 °C likely to have involved increased ATP. In contrast, the decrease in extractable ATP effected by growth in LiCl was significantly different from the control values at the 98% confidence limit (Student's *t*-test), suggesting that lithium inhibition of growth and dimorphic transition has its basis at least in part in an energetic restriction.

Fig. 4. Yeast growth in 200 mM sodium gluconate (○, ●) or LiCl (□, ■) at pH 5.5 (open symbols) or pH 8.0 (filled symbols) and 27 °C. Growth was determined as the increase in optical density (see Methods). The μ values (mean $\pm$ SE), estimated for the 4–6 h interval, were: 0.17 ± 0.01 , sodium gluconate pH 5.5 ($n = 4$); 0.09 ± 0.01 , sodium gluconate pH 8.0 ($n = 3$); 0.09 ± 0.01 , LiCl pH 5.5 ($n = 4$); 0 , LiCl pH 8.0 ($n = 3$).

The retention of the yeast form at pH 8.0/27 °C in the presence of either 200 mM sodium gluconate or LiCl allowed interaction of salt and pH to be examined in a single morphic form. The effect of alkaline and acidic pH (8.0 and 5.5) on yeast growth at 27 °C is shown in Fig. 4. Clearly alkaline pH restricted growth in both salts. The effect was also observed in 200 mM NaCl prior to germ tube formation (data not shown). Again the LiCl results are difficult to interpret in straightforward terms of putative Na^+ transport because of Li^+ cytotoxicity, but the similarity to the Na^+ response is

striking. The Na^+ inhibition of germination which occurred only at high pH was reflected in yeast growth; Na^+ inhibition was pH-dependent. This strongly resembles the response of *S. pombe* to growth in Na^+ at high pH (Jia *et al.*, 1992) and suggests the presence of a PM Na^+/H^+ antiporter which would be thermodynamically compromised for Na^+ extrusion at alkaline pH. In this sense, the observed Na^+ inhibition of germ tube formation at pH 8.0 could be explained in part by Na^+ cytotoxicity due to impaired extrusion capacity under Na^+ stress.

Effects of growth in Na^+ salts on PM H^+ -ATPase activity *in vitro*

If the pre-germinative pH_i increase reported in *C. albicans* (Stewart *et al.*, 1988) was the consequence of increased PM H^+ -ATPase activity then it is feasible that retardation/inhibition of the transition by Na^+ and gluconate could be the result of decreased enzyme specific activity or decrease in enzyme protein abundance. The latter has not been tested here but Table 2 shows the effects of 5 h growth in 200 mM Na^+ salts (pH 5.5, 27 °C) on PM ATPase hydrolytic activity, measured in permeabilized yeast cells. Under control growth conditions, vanadate-sensitive (i.e. PM) ATPase activity was slightly higher than the range reported by Kaur & Mishra (1991). Use of sodium vanadate to identify PM ATPase activity yielded 30 mM Na^+ in the assay. To differentiate between vanadate and Na^+ effects in this instance, 30 mM NaCl was added to the control assay; there were no statistically significant differences in total ATPase activities with or without Na^+ for any growth conditions (Table 2). This indicated that the inhibition by sodium vanadate was effected by the anion and identified the PM ATPase component. Further, 30 mM Na^+ had no inhibitory effect even though the resultant $\text{Na}^+:\text{K}^+$ ratio (1.7) was an order of magnitude

Table 2. *In vitro* ATPase hydrolytic activity in permeabilized yeast cells grown at pH 5.5 and 27 °C under control and test conditions

Cells were harvested at 5 h. Total ATPase specific activity was that recorded in the absence of inhibitors; vanadate-sensitive ATPase was activity inhibited by 10 mM sodium orthovanadate (76 % mean inhibition of total for control-grown cells). In the presence of vanadate, azide, nitrate and molybdate, all activity was abolished (concentrations in Methods). In controls for the Na^+ component of sodium orthovanadate, 30 mM NaCl was added to the assay medium with no inhibitors. Results are means $\pm$ SE of three independent trials, each constituting triplicate samples per treatment.

Growth medium	ATPase activity [nmol P_i (mg protein) $^{-1}$ min $^{-1}$]		
	Total ATPase	Vanadate-sensitive ATPase	Total ATPase; 30 mM NaCl assay
Control	566 $\pm$ 65	430 $\pm$ 55	569 $\pm$ 70
Sorbitol	613 $\pm$ 73	503 $\pm$ 58	617 $\pm$ 71
200 mM NaCl	683 $\pm$ 38	574 $\pm$ 46	701 $\pm$ 47
200 mM sodium gluconate	550 $\pm$ 43	420 $\pm$ 33	558 $\pm$ 31

Fig. 5. Initial rates of glucose-induced medium acidification. Yeast cells were grown for 5 h in control medium (○), plus 200 mM NaCl (●) or plus 200 mM KCl (□), pH 5.5, 27 °C. Assays were conducted in simple buffer with no salt additions (pH 4.5–8.0) as described in Methods. Results are means $\pm$ SE from three independent trials. Inset: superimposed examples of glucose-induction acidification traces from (1) control-grown and (2) NaCl-grown yeast cells assayed at pH 8.0; (3) control-grown cells with 1 mM sodium azide in assay medium (pH 8.0). The arrow indicates addition of glucose (final concentration 2 mM).

greater than that estimated for the cytoplasm of another *Candida* species, *C. tropicalis* (around 0.1 during growth in 250 mM NaCl; Garcia *et al.*, 1997).

There were no statistically significant differences in vanadate-sensitive PM ATPase specific activity as a function of growth in NaCl or sodium gluconate versus either the null addition or osmotic control. As the duration of salt exposure in this test (5 h) exceeded the lag period for germ tube formation (as shown in Fig. 2), it seems unlikely that inhibition of the dimorphic transition by either Na⁺ or gluconate has its basis in a reduction of H⁺-ATPase specific activity induced by salt growth. However, it should be borne in mind that vanadate sensitivity of ATP hydrolysis may not be effective in discriminating between ATP hydrolysis by a PM H⁺-translocating and an Na⁺-translocating ATPase, as both belong to the same class of ion pump (Haro *et al.*, 1991). If *C. albicans* possessed a PM Na⁺-ATPase then it remains feasible (however unlikely) that during salt growth the relative proportion of the Na⁺-ATPase increases (for constant total ATPase activity) against the H⁺-ATPase, which could compromise cytosolic alkalization. However, if this were the case then Na⁺ inhibition of germ tube formation at pH 5.5 and 37 °C would be anticipated and this was not observed. Overall, there is no clear case in favour of linking Na⁺ and gluconate effects on germ tube formation to the specific activity of PM H⁺-ATPase.

Table 3. Effect of salts and inhibitors on initial rates of glucose-induced medium acidification at pH 8.0

Yeast cells were grown in control medium at pH 5.5 and 27 °C for 5 h. Details of the assay are given in Methods. Results are means $\pm$ SE of three independent trials.

Assay medium*	Initial rate [nmol H ⁺ (mg fresh wt) ⁻¹ min ⁻¹]
Control	46 $\pm$ 2
1 mM NaCl	21 $\pm$ 4
1 mM sodium gluconate	9 $\pm$ 1
1 mM Cl ⁻	30 $\pm$ 6
10 mM Cl ⁻	19 $\pm$ 3
1 mM gluconate	11 $\pm$ 3
10 mM gluconate	10 $\pm$ 2
1 mM NEM	11 $\pm$ 2
0.1 mM niflumic acid	28 $\pm$ 7
0.1 mM DIDS	18 $\pm$ 2

* DIDS, 4,4'-diisothiocyanato-stilbene-2,2'-disulphonic acid; NEM, N-ethylmaleimide.

Rates of glucose-induced medium acidification; effect of growth with Na⁺

The initial rate of glucose-induced medium acidification by yeast has been proposed as a simple diagnostic of glucose-stimulated PM H⁺-ATPase activity (Serrano, 1980), but its use at varying pH is complicated by possible differential rates of organic acid efflux and activities of other transporters involved in regulating membrane voltage and pH_i. As there are so many unknown and uncontrolled parameters in the system, results from this assay are liable to overinterpretation but may be of consequence to the mechanism of cytosolic alkalization which precedes germ tube growth. In this study, glucose-induced medium acidification was inhibited by 1 mM azide (Fig. 5), which at least confirms that extrusion of H⁺ equivalents was ultimately energy-dependent and implicates the operation of a PM H⁺-ATPase. Acid extrusion was also inhibited (Table 3) by the thiol agent N-ethylmaleimide, which is known to inhibit the fungal PM H⁺-ATPase (Brooker & Slayman, 1982) and depolarize fungal PM membrane voltage (Davies *et al.*, 1990).

Fig. 5 also shows that prior growth of yeast cells in 200 mM NaCl at pH 5.5/27 °C resulted in impaired glucose-induced acidification (in the absence of external salts), increasingly apparent as assay pH was increased from 4.5 to 8.0. As shown by the result for growth in KCl, this was an Na⁺-specific effect. Without more detailed information on the physiological status of the effect can only be speculated on but it is significant that this was the only case thus far in which high pH and Na⁺ could be linked to inhibition of a fundamental process relevant to dimorphism. If, from the study on PM ATPase activity, direct Na⁺ inhibition of pumps can be discounted then Na⁺ effects on other transporters or

the bilayer itself must be invoked. It is perhaps significant that in the salt-tolerant yeast *Zygosaccharomyces rouxii*, PM lipid composition is a component of Na^+ tolerance (Yoshikawa *et al.*, 1995). Any change in bilayer composition induced by salt growth would be envisaged to affect the kinetics of integral transport proteins. A component of the Na^+ effect on germination at 27 °C could be due to bilayer properties affecting transport systems which mediate cytosolic alkalization, with amelioration of the effect at 37 °C through bilayer fluidity changes.

Effect of Na^+ and anions on acidification rates

To examine further the effects of Na^+ and anions on acid extrusion by the yeast form grown under control non-permissive conditions, Na^+ salts were incorporated into the assay medium at pH 8.0. The results in Table 3 clearly show that even at 1 mM, both NaCl and sodium gluconate were effective at reducing acid extrusion rate. The same pattern of salt effect emerged as for germ tube inhibition at similar temperature and pH 8.0; i.e. sodium gluconate was more inhibitory than NaCl. When the anions alone were examined, a marked difference between Cl^- and gluconate was observed. Gluconate inhibition appeared already saturated at 1 mM and at this concentration comparison with sodium gluconate implicates only a small inhibitory component for Na^+ in the salt effect. In contrast, 1 mM Cl^- permitted greater acidification rates than 1 mM NaCl, suggesting an inhibitory role for Na^+ . The mechanistic basis for Na^+ inhibition of acidification is not clear, but presumably in the absence of K^+ , Na^+ may easily enter the cell through K^+ -uptake systems. What effect this would have on voltage and cellular processes over the assay time-scale is unknown. Increasing Cl^- concentration 10-fold increased inhibition of acidification.

Placing these results into a model of cellular processes which in turn relate to the salt effects on germination is, as stated previously, confounded by unknowns and variables. However, some analogies with animal cells may be drawn. Animal cell PM energized by a H^+ pump often contains both Cl^- exchangers (which mediate extrusion of alkaline equivalents and regulate pH_i) and Cl^- channels (which permit continued pump action by dissipating voltage); e.g. *Leishmania major* promastigote PM contains this transport array (Viera *et al.*, 1995). In the *Candida* acidification assay under control conditions, the high rate of acid extrusion ($46 \text{ nmol H}^+ \text{ mg}^{-1} \text{ min}^{-1}$) could have been generated by the H^+ pump acting in parallel with a Cl^- channel; the absence of external Cl^- would establish a steep gradient for efflux of this ion and promote pump activity. In addition, envisaging an initial near neutral pH_i (pH_i can remain stable at high pH; Rabaste *et al.*, 1995), the transmembrane pH gradient could reverse a putative Cl^- - OH^- exchanger, resulting in further medium acidification. The existence of a Cl^- exchanger has already been proposed to explain discrepancies between PM H^+ -ATPase activity and rates of pH_i change (Kaur &

Mishra, 1991). Increasing external Cl^- would reduce the magnitude of the outwardly directed Cl^- gradient pertinent to both putative anion transporters and reduce their rates, thus reducing in turn both pump- and exchanger-mediated acidification rates. Placing gluconate in the assay would block the anion transport pathways, eliminating acidification by the exchanger and reducing pump activity by blocking full control of membrane voltage.

To test for a Cl^- transport component in glucose-induced acidification, the effects of the Cl^- channel blocker niflumic acid (Garrill *et al.*, 1996) and stilbene Cl^- transport inhibitor 4,4'-diisothiocyano-2,2'-disulphonic acid (Viera *et al.*, 1995) were tested; results are shown in Table 3. Again, interpretation of results must be cautious as the specificity of the inhibitors in this system is unknown (the specificity of niflumic acid in plant systems has been questioned; Garrill *et al.*, 1996), but it may be concluded tentatively that the inhibitory effects of these agents on acidification is indicative of a role for Cl^- in pump and pH_i regulation.

Towards a model for Na^+ and anion regulation of yeast to hypha transition

It is premature but nevertheless instructive to relate the glucose-acidification results to germination profiles, on the assumption that external acidification may be related ultimately to internal alkalization. Inhibition of germination at pH 8.0 by gluconate may be explained by a simple block of the putative Cl^- transport systems (channel and/or exchanger) necessary to permit PM H^+ -ATPase activity and increase pH_i . It is predicted that gluconate would inhibit cytosolic alkalization; this is certainly the case with *Leishmania*, where replacing external Cl^- with gluconate at alkaline pH depresses the extent of cytoplasmic pH_i increase (Viera *et al.*, 1995). Does this model explain gluconate stimulation of germination at pH 5.5? At acidic pH it is proposed that the Cl^- -anion exchanger would be operating normally, i.e. Cl^- influx coupled to extrusion of alkaline equivalents. External gluconate would inhibit this activity, perhaps causing a sufficient increase in pH_i to stimulate germination. It is also anticipated that gluconate block of the putative Cl^- efflux channel would in part inhibit acid extrusion by the pump; this was confirmed experimentally by performing the glucose acidification assay at pH 5.5. For yeast cells grown and assayed under control conditions, the rate of medium acidification was (mean $\pm$ SE) $6 \pm 1 \text{ nmol H}^+ \text{ mg}^{-1} \text{ min}^{-1}$ ($n = 3$). Addition of 10 mM gluconate reduced acidification to $4 \pm 1 \text{ nmol H}^+ \text{ mg}^{-1} \text{ min}^{-1}$ ($n = 3$). Therefore, the model predicts that at acid pH the effect of blocking a Cl^- exchanger would have more profound consequences to pH_i than restricting the PM H^+ -ATPase.

The possible role of Na^+ in regulation of yeast/hypha transition is less clear as there are multiple sites at which Na^+ could act. This study has produced evidence for Na^+ -specific retardation and inhibition of germ tube formation at high pH, which can be offset to some

extent by increasing temperature. There is also evidence for high pH reducing Na⁺ tolerance of yeast growth, implicating the existence of a PM Na⁺–H⁺ antiporter. By analogy with animal cells, this putative system could be involved in pH_i control.

What is clear now is that the activity of the PM H⁺-ATPase in dimorphism should not be regarded as of paramount importance; there are other transport proteins implicated in the response that are yet to be characterized. Studies on intracellular pH in this yeast must pay close attention to the anion conditions used, indeed the effect of extracellular anions on pH_i regulation must be studied directly. Furthermore, there is a case for further examination of the mechanism of Na⁺ extrusion in *C. albicans*.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the gift of strain CAI-4 from Professor Neil Gow (University of Aberdeen, UK) and advice on data analysis from Dr Neil Mackay (JSC International, Harrogate, UK), Dr Till Jelitto (University of York) and Dr Chris Gilligan (University of Cambridge). For financial support we thank the Wellcome Trust (vacation studentship to S.L.), the Royal Society of London (University Research Fellowship to J.M.D.), the University of Cambridge and the BBSRC (grant 87/PO4954 awarded to J.M.D.).

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Received 23 May 1997; revised 13 August 1997; accepted 18 August 1997.