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Amiloride and 5-(*N*-ethyl-*N*-isopropyl)amiloride inhibit medium acidification and glucose metabolism by the fission yeast *Schizosaccharomyces pombe*

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We have investigated the mechanism by which amiloride and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) inhibit glucose-stimulated medium acidification in the fission yeast *Schizosaccharomyces pombe*. The addition of glucose to an unbuffered suspension of cells results in the extrusion of acid. This process was inhibited by diethylstilbestrol (DES), an inhibitor of the H^+-ATPase (IC_{50} 71 μM), and also by amiloride (IC_{50} 824 μM) and EIPA (IC_{50} 203 μM). The presence of 100 mM NaCl reduced the degree of inhibition observed for amiloride and EIPA, but had no effect on inhibition by DES. *N*-Methylglucosamine partially protected the cells against the effect of amiloride, but choline chloride did not, suggesting that sodium may be important in the action of amiloride. To establish the site of action of amiloride and EIPA, ATP hydrolysis assays were performed on isolated plasma membranes. H^+-ATPase activity was inhibited by orthovanadate, but not by amiloride or EIPA. However, both amiloride and EIPA were found to inhibit the incorporation of radioactivity from labelled glucose in *S. pombe*, with IC_{50} values of 879 and 272 μM for amiloride and EIPA respectively. Again, 100 mM NaCl was found to reduce the effectiveness of inhibition. Amiloride had no effect on the uptake of 2-deoxyglucose under the same conditions, indicating that amiloride does not inhibit the glucose transporter. We propose that amiloride and EIPA disrupt glucose-induced acidification by inhibiting glucose metabolism.

Introduction

The budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* contain a highly conserved plasma membrane H^+-ATPase [1,2]. The hydrolysis of ATP by the enzyme results in the extrusion of protons from the cytoplasm, creating an electrochemical proton gradient across the cytoplasmic membrane. The H^+-ATPase is thus important in both the regulation of internal pH and the energy-dependent uptake of various metabolites.

Glucose activates the plasma membrane H^+-ATPase by a mechanism which is not understood. In *S. cerevisiae*, glucose addition has been shown to cause a shift in the pH optimum of the enzyme to a more alkaline pH, to increase the affinity of the enzyme for ATP, and to increase the sensitivity of the enzyme to the inhibitor orthovanadate [3]. Glucose regulation of the H^+-ATPase in *S. cerevisiae* depends upon the final eleven residues at the carboxyl terminal of the H^+-ATPase [4,5], which carry a potential site for phosphorylation by calmodulin-dependent protein kinase [5]. Phosphorylation of the enzyme appears to be essential for its activity [6], and it seems probable that glucose-induced phosphorylation of the enzyme is responsible for activation in *S. cerevisiae*. The control of the H^+-ATPase in *S. pombe* is less well understood, although the structural and functional similarities between the H^+-ATPases in the two species of yeast suggests that they may have similar control mechanisms. However, the events which occur between glucose uptake and H^+-ATPase activation are not known for either species.

Glucose transport in *S. pombe* has not been studied in great detail. Hofer and Nassar [7] demonstrated that the low-affinity glucose carrier is a H^+-symporter, in contrast to glucose transport in *S. cerevisiae*, which is

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Abbreviations: DES, diethylstilbestrol; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; IC_{50}, inhibitory constant; DMSO, dimethyl sulphoxide; 2-DOG, 2-deoxyglucose.

by facilitated diffusion [8]. Glucose uptake in *S. pombe* is inhibited by *N,N'*-dicyclohexylcarbodiimide, an inhibitor of the plasma membrane H^+-ATPase.

We have recently investigated the effects of amiloride and EIPA on glucose-stimulated plasma membrane H^+-ATPase activity in *S. cerevisiae* [9]. The addition of glucose to a stirred suspension of yeast resulted in an acidification of the medium. This appears to be a very complex process, with acid coming from a number of sources, including carbonic acid, the extrusion of organic acids, and the activation of the plasma membrane H^+-ATPase [10]. The external pH was found to decrease from 7 to 5.5 when glucose was added. Subsequent addition of KCl elicited a further extrusion of protons, probably mainly through the ATPase [11]. Preincubation of the yeast cells with diethylstilbestrol (DES), an inhibitor of the ATPase [12], drastically reduced both stages of acidification. Amiloride and EIPA also inhibited medium acidification, by a mechanism which was not understood. Here, we show that amiloride and EIPA have similar effects in *S. pombe* and we examine the mechanism by which these effects occur.

Materials and Methods

Materials

Diethylstilbestrol, amiloride hydrochloride dihydrate and sodium orthovanadate were from Sigma (St. Louis, MO, USA). EIPA was synthesized as previously described [13]. [U-^{14}C]Glucose (2.9 mCi/mmol) and 2-deoxy-D-[1-^{3}H]glucose (11.0 Ci/mmol) were from Amersham Canada (Oakville, Ontario). All other materials and reagents were from BDH or Fisher, and were of analytical grade or higher.

The strain of *Schizosaccharomyces pombe* used in this study, 38436, has the phenotype h^- ura4–294, and was obtained from the American Type Culture Collection, MD, USA.

Growth and isolation of yeast

Cells were grown overnight in YD medium (5% glucose and 1% yeast extract) at 30°C in an orbital incubator (160 rpm). The cells were harvested at mid log phase ($2 \cdot 10^7$ cells/ml) by centrifugation at $3000 \times g$ for 5 min, washed twice with 25 ml water and resuspended to $1 \cdot 10^8$ cells/ml with water. All operations were conducted at 20°C.

Measurement of changes in external pH

To measure changes in external pH caused by the addition of glucose to yeast cells, a procedure similar to that used previously for *Saccharomyces cerevisiae* was employed [9]. After incubation on ice for 2 h, the cells were washed once with distilled water (25 ml) and resuspended in water to $2 \cdot 10^8$ cells/ml. Aliquots (1 ml) were placed in a stirred cuvette containing distilled water (1 ml), and the pH was measured using a Fisher Scientific Accumet 925 pH meter. Data was collected with an Apple Macintosh SE/30 computer. Proton extrusion was initiated by the addition of 11 mM glucose.

Determination of buffering capacity

To determine buffering capacities, cells were prepared as for measurement of proton extrusion. Aliquots (2 μl) of 1 mM HCl were added to the suspension ($2 \cdot 10^8$ cells/ml). The pH of the suspension after each addition was determined as above. Buffering capacity was calculated from a plot of pH against nmol HCl added [14]. The buffering capacities of 1 mM solutions of DES, amiloride and EIPA were determined by the same procedure.

Isolation and partial purification of plasma membranes

An overnight culture of *S. pombe* (500 ml) was harvested at mid-log phase, and washed twice with 400 ml of a buffer containing 10% sorbitol and 20 mM Tris (pH 7.0). The cells were incubated with β-mercaptoethanol (0.5 μl/ml) and zymolyase (ICN; 2 mg/ml) at 30°C for 3–4 h, at which time more than 90% of the cells were spheroplasted. The spheroplasts were washed twice with sorbitol buffer (30 ml), resuspended in 2 ml sorbitol buffer and transferred to a 40 ml Dounce homogeniser. Buffer (20 mM Tris, pH 7.0) containing a cocktail of protease inhibitors [15] was added to a final volume of 30 ml, and the cells were lysed by homogenisation (10 strokes). Unbroken cells and debris were removed by centifugation at $5000 \times g$ for 5 min at 4°C. The cell membranes were isolated by centrifugation at $60\,000 \times g$ for 60 min at 4°C (Beckman L80 ultracentrifuge). The pellet was resuspended in 10 ml of a buffer containing 20% glycerol, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 μl/ml β-mercaptoethanol and 10 mM Tris (pH 7.5). The membranes were separated on a discontinuous sucrose gradient (53.5 and 43.5% sucrose in Tris (pH 7.2); 9 ml and 18 ml, respectively) by centrifugation at $24\,000 \times g$ for 90 min at 4°C (SW24 swingout rotor), as described by Serrano [3]. Membranes at the interface were collected, washed twice with a buffer (20 ml) containing 5 mM $MgCl_2$, 1 mM EDTA, 50 mM Tris (pH 7.0) (MET buffer), and resuspended in MET buffer to 10 mg protein/ml. Protein was determined by the method of Bradford [16], with bovine serum albumin as standard.

Assay of ATP hydrolysis

H^+-ATPase activity was determined by incubating membranes (50 μg protein/ml) in MET buffer at 30°C for 5 min. ATP (2 mM) was added, and incubation continued for 15 min. At this time, phosphate production was determined by the colorimetric method of

Dufour et al. [17], using disodium hydrogen phosphate to prepare a calibration curve. Inhibitors were added to the membranes 5 min prior to the ATP, from concentrated stock solutions of inhibitors dissolved in dimethyl sulphoxide. Dimethyl sulphoxide had no effect on H^+-ATPase activity at the concentrations used (less than 1% of the total volume).

Glucose uptake

An overnight culture of *S. pombe* was harvested and washed twice with water. The cells were resuspended to $1 \cdot 10^8$ cells/ml with water, and kept on ice. Aliquots (1 ml) were withdrawn and incubated with [^{14}C]glucose (11 mM; 0.01 μCi/ml) or 2-[^{3}H]deoxyglucose (11 mM; 1 μCi/ml). After 5 min, three times 300 μl aliquots were removed and filtered through Millipore HA filters (0.45 μm pore size). The filters were washed twice with water (3 ml). EcoLite™ scintillation fluid (ICN Biomedicals) was added to each sample prior to counting. Inhibitors were introduced 5 min prior to the addition of glucose.

Statistical analysis

Where appropriate, data are presented as the mean ± S.E.

Results and Discussion

Measurements of the pH of media indicate that cells suspended at high concentrations in distilled water do not extrude a significant number of protons when unchallenged (Fig. 1, inset). The initial media pH of a suspension of cells varied between 6 and 7. When glucose (11 mM final concentration) was added, the cells extrude acid at a high rate (5.2 ± 0.5 μmol H^+/min/l (from 13 experiments)). The cells were able to reduce external pH to between 4.5 and 5 within 10 min after the addition of glucose, at which point external pH rose slightly and then stabilized (Fig. 1, inset). The very reproducible rise in pH 10–15 min after the addition of glucose is probably due to the depletion of glucose in the cells. Under such conditions, organic acids in the medium are transported back across the plasma membrane, resulting in a slight alkalinisation of the medium [10]. The change in external pH upon the addition of glucose is characteristic of yeast cells. Under the conditions employed, the acid extruded could be due to the action of the plasma membrane H^+-ATPase, the excretion of acidic glycolytic intermediates and to the production of carbonic acid by the diffusion of CO_2 produced by glucose metabolism [10]. Similar experiments where 2-DOG was added instead did not result in medium acidification (not shown), indicating that glucose metabolism is required for this process.

For calculating proton fluxes across the yeast cytoplasmic membrane, the buffering capacities of the cell

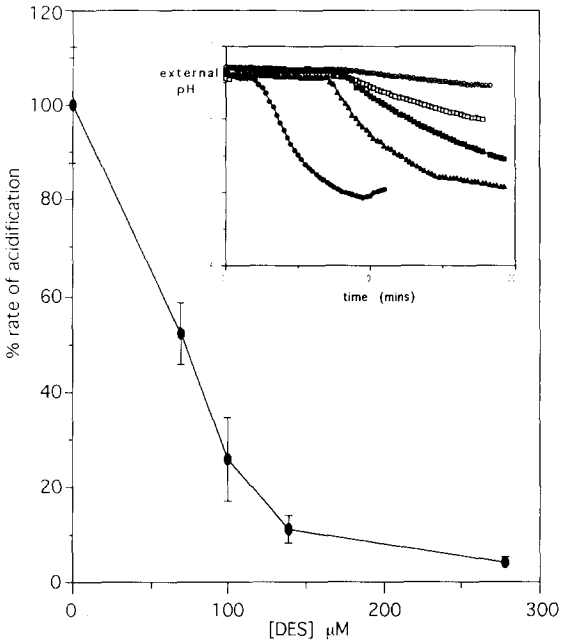

Fig. 1. Inhibition of acidification by diethylstilbestrol. Inset: external pH was determined as described in Materials and Methods. Cells were incubated in the absence (●) or presence of 69 (▲), 100 (■), 139 (□), or 278 μM (○) DES for 15 min prior to the addition of glucose. In the absence of inhibitor, the addition of glucose caused a significant acidification of the suspension. Preincubation of cells in the presence of DES caused an decrease in the rate and extent of acidification. The rate of proton extrusion was determined from the slope of the line after the addition of glucose multiplied by the buffering capacity of the suspension at each concentration of inhibitor. Values are shown relative to the rate of proton extrusion in the absence of inhibitor (main panel).

suspension and of the compounds used were determined, as described in Materials and Methods. Cells alone ($2 \cdot 10^8$ cells/ml) had a buffering capacity of 12.7 ± 1.6 μmol H^+/l per pH unit (6). The buffering capacities of 1 mM solutions of DES (4.6 ± 0.1 μmol H^+/l per pH unit), amiloride (5.5 ± 0.2 μmol H^+/l per pH unit), and EIPA (39.2 ± 2.9 μmol H^+/l per pH unit) were used to calculate the buffering capacities of the suspension at each concentration of inhibitor used.

Preincubation of the cells with DES inhibited acid extrusion (Fig. 1). Increasing the concentration of DES from 0 to 278 μM resulted in the inhibition of acidification, with an IC_{50} of 70 ± 6 μM (3). The near completeness of the inhibition of acidification by DES is surprising, as the plasma membrane H^+-ATPase is not thought to play a major role under the conditions used. The IC_{50} reported here is similar to previous results using purified H^+-ATPase from *S. pombe* (27 μM; [18]). The inhibition of acid extrusion by DES may be due to a direct effect on the H^+-ATPase, reducing the magnitude of the proton gradient, and hence glucose uptake.

Both amiloride and the amiloride analog EIPA inhibited medium acidification by *S. pombe* (Fig. 2), with

IC_{50} values of 824 ± 22 μM (3) and 203 ± 13 μM (3) for amiloride (Fig. 2a) and EIPA (Fig. 2b), respectively. Since these compounds have been shown to affect Na^+-dependent transport processes by competing for Na^+-binding sites [19], we investigated the effect of NaCl on the inhibition of acid extrusion. The results of inhibitory studies with amiloride, EIPA and DES in the presence of 100 mM NaCl are shown in Fig. 3. The presence of NaCl (100 mM) almost completely reversed the inhibition of proton extrusion by amiloride and EIPA, but had no effect on the action of DES (Fig. 3a). Thus, amiloride and EIPA may act by a different mechanism than DES. Sodium chloride alone had no effect on proton extrusion (Fig. 3a). Variations in ionic strength could affect the possible interactions between amiloride and binding sites on a potential amiloride uptake system. Increasing the salt concentration could screen electrostatic interactions, effectively reducing inhibitor binding and hence entry into the

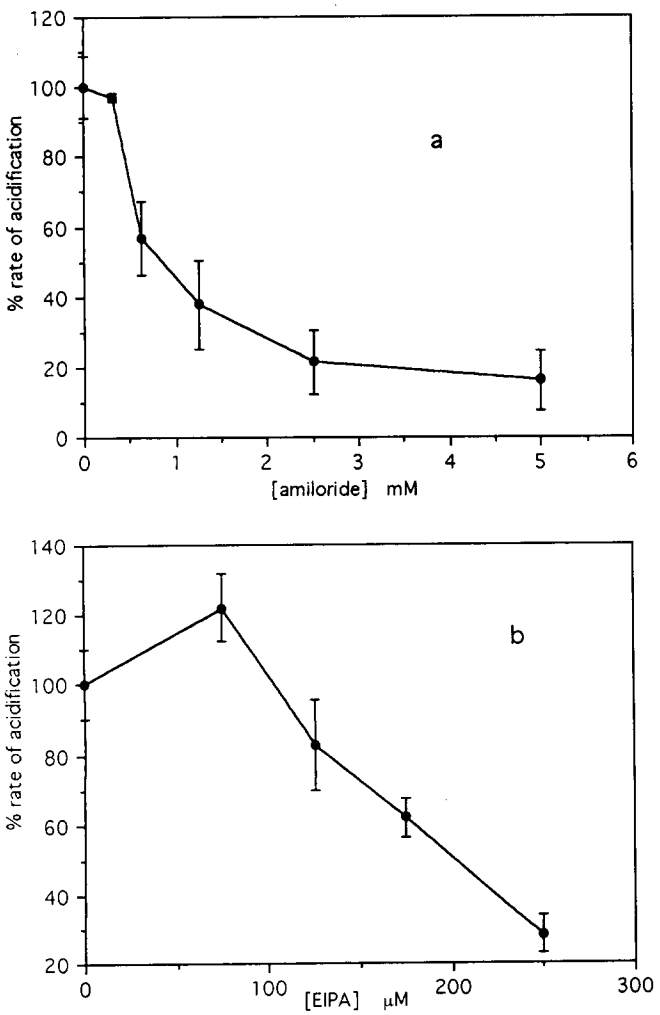

Fig. 2. Inhibition of acidification by amiloride and EIPA. Data was obtained as described in Materials and Methods, and processed as described in the legend to Fig. 1. a, amiloride; b, EIPA.

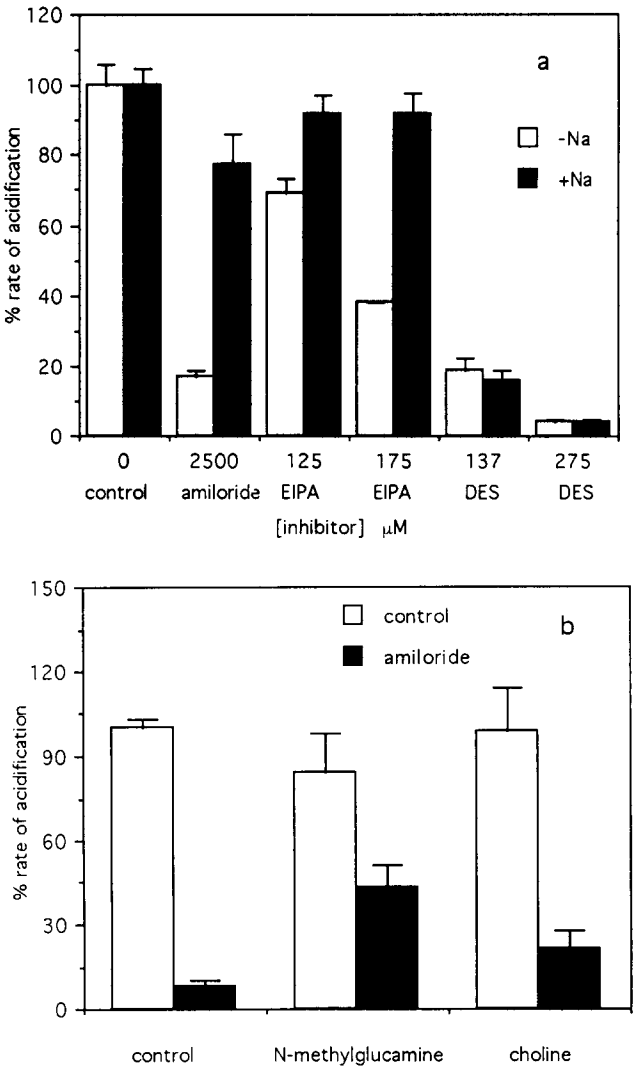

Fig. 3. Effect of cations on action of DES, amiloride and EIPA. (a) Effect of sodium chloride on inhibition of medium acidification by amiloride, EIPA and DES. Cells were incubated in the presence (■) or absence (□) of 100 mM NaCl, and either in the absence or presence of inhibitor, as described. (b) Effect of other cations on inhibition of acidification by amiloride. Cells were incubated in the presence (■) or absence (□) of 5 mM amiloride, in medium containing 100 mM *N*-methylglucamine or choline, as indicated.

cell. To determine whether the protective effect of NaCl was due to a general ionic effect, or specifically to the presence of sodium, further experiments with different cations were performed. The presence of 100 mM *N*-methylglucamine had a slight inhibitory effect on the rate of medium acidification (Fig. 3b). Amiloride (5 mM) was less effective at inhibiting acidification in the presence of this cation, although the extent of protection was not as great as seen with sodium (Fig. 3b). Choline chloride (100 mM) afforded little protection from the action of amiloride (Fig. 3b). Thus, it appears that the protective properties of sodium are not due to a general ionic effect. The mechanism by which sodium, and to a lesser degree *N*-methylgluca-

mine, reduce the effectiveness of amiloride is not known. One possible effect of sodium could be on the mechanism by which amiloride enters the cells.

In mammalian cells, both amiloride and EIPA are known to act on several sodium-dependent transport processes [19]. At higher concentrations, these compounds also inhibit other systems, such as adenylate cyclase [19]. Amiloride and EIPA both inhibited acid fluxes across the yeast cytoplasmic membrane induced by the addition of glucose. The IC_{50} values for amiloride and EIPA reported here (824 and 203 μM, respectively) are within the range observed for inhibition of several transport processes by these compounds [20]. There are a number of possible sites of action for amiloride and EIPA which could account for the experimental observations. These include a direct effect on the H^+-ATPase or an inhibition of the processes responsible for activating the H^+-ATPase, both of which would affect the electrochemical proton gradient required for glucose uptake; inhibition of glucose uptake by interaction with the transporter; or inhibition of glucose metabolism. To localise the site of action of amiloride and EIPA, we examined the effects of these compounds on ATP hydrolysis and glucose uptake.

A direct effect on the H^+-ATPase is not unlikely, as another P-type ATPase, the Na^+/K^+-ATPase, has been shown to be inhibited by millimolar concentrations of amiloride [21,22]. We therefore measured plasma membrane H^+-ATPase activity in the absence of inhibitors, or in the presence of different concentrations of orthovanadate, amiloride or EIPA. The data presented in Fig. 4 show that H^+-ATPase activity was inhibited by orthovanadate, with an IC_{50} of 50 ± 4 μM. EIPA had no effect on ATP hydrolysis over the concentration range studied (0–500 μM; Fig. 4). Amiloride also had no effect on ATP hydrolysis at up to 2.5 mM (not shown). Thus, inhibition of glucose-stimulated acid secretion by amiloride and EIPA is not due to inhibition of the H^+-ATPase itself. We did not look for an indirect effect, such as inhibition of ATPase-activating kinases, and thus some involvement of the ATPase cannot be ruled out at this stage. However, as acid extrusion was also inhibited in *S. cerevisiae* [9], which does not require a protonmotive force for glucose uptake, the ATPase is probably not involved.

Glucose accumulation was characterised as described in the Methods section, under conditions designed to mimic proton extrusion experiments as closely as possible. The accumulation of glucose and 2-DOG tracer was determined at different times (Fig. 5a). Glucose accumulation reached a peak at approx. 10 min under the conditions used. The accumulation of 2-DOG, however, reached a lower maximum within 1 min. The difference in tracer uptake between glucose and 2-DOG may be due to the lack of metabolism of 2-DOG, leading to an increased concentration within

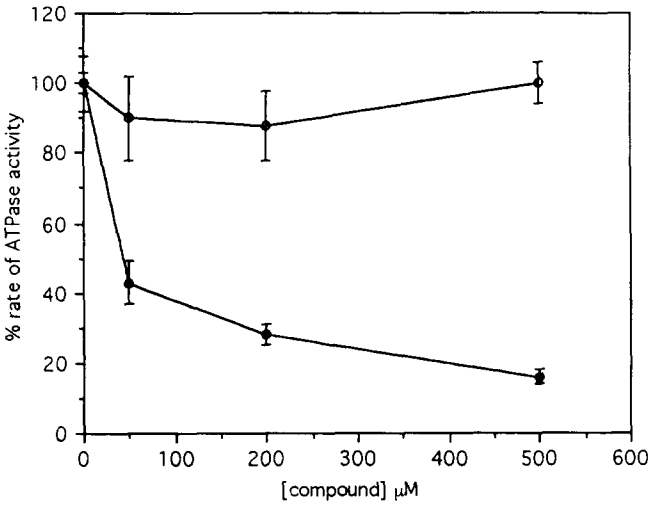

Fig. 4. Effect of orthovanadate and EIPA on ATP hydrolysis by partially purified plasma membranes from *S. pombe*. ATP hydrolysis was determined as described in Materials and Methods, in the presence of either orthovanadate (●) or EIPA (○).

the cells. Glucose, however, is rapidly metabolised within the cells, which leads to further uptake and accumulation of labelled metabolites in the cell. The observation that [^{14}C]glucose and its metabolites are accumulated only 4–5-fold more than 2-DOG may be due to the loss of glucose label in the form of $^{14}CO_2$ and other ^{14}C-labelled metabolites.

The compounds tested were added from stock solutions made up in dimethyl sulphoxide (DMSO). We found that DMSO had a positive effect on glucose accumulation (Fig. 5b), and so suitable controls were performed for each experiment. The data presented in Fig. 5 show that amiloride (Fig. 5c) and EIPA (Fig. 5d) reduce glucose accumulation in *S. pombe*, with IC_{50} values of 879 ± 21 μM and 272 ± 18 μM, respectively. These values are comparable to the inhibitory constants obtained in acid extrusion experiments. The simplest interpretation of these results could be that glucose transport is inhibited by amiloride and EIPA. However, our experiments with 2-DOG showed that amiloride does not affect the transport process. 2-DOG is taken up by the same transporter as glucose, with very similar affinity [23]. Incubation with even high concentrations of amiloride had no significant effect on the quantity of 2-DOG within the cell (Fig. 5c). Thus, amiloride and EIPA must affect a process subsequent to glucose uptake, rather than inhibiting the glucose transporter itself. This experiment also argues against an involvement of the ATPase in amiloride action, as a reduction in the protonmotive force would be expected to affect the uptake of 2-DOG. Complete inhibition of glucose accumulation was not observed, even at high concentrations of compound. This is compatible with a mechanism of inhibition where glycolysis is affected. The initial uptake of glucose into the cell would not be

affected by amiloride or EIPA. However, if glycolysis is inhibited, glucose metabolites would rapidly accumulate, until the equilibrium for further glucose uptake is unfavourable. Thus, glucose accumulation in the presence of amiloride would be predicted to show very similar characteristics to the accumulation of 2-DOG. This is approximately the case observed here. In the absence of inhibitor, labelled glucose tracer is accumulated 4–5-fold more than 2-DOG under the conditions used. However, when a maximal inhibitory quantity of amiloride is present, the quantity of radiolabelled glucose within the cell decreases five fold to a level approximately equivalent to that of 2-DOG.

As with acidification experiments, inhibition of glucose accumulation by amiloride was much reduced by the inclusion of 100 mM sodium chloride (Fig. 6). Glucose accumulation was not affected by the addition of NaCl in the absence of inhibitor (Fig. 6). Pretreatment of the cells with 1.25 and 2.5 mM amiloride (Fig. 6) caused substantial inhibition of glucose accumulation, as before (Fig. 5). However, when NaCl was included, inhibition by amiloride was practically abolished, giving roughly the same degree of protection as was found with the proton extrusion measurements. The results of the experiments with different cations could be reflective of an effect on the mechanism by which amiloride and EIPA enter the cell, rather than of their inhibitory mechanism. The observation that the potency of inhibition by EIPA is greater than that of amiloride is interesting. EIPA and other 5-substituted

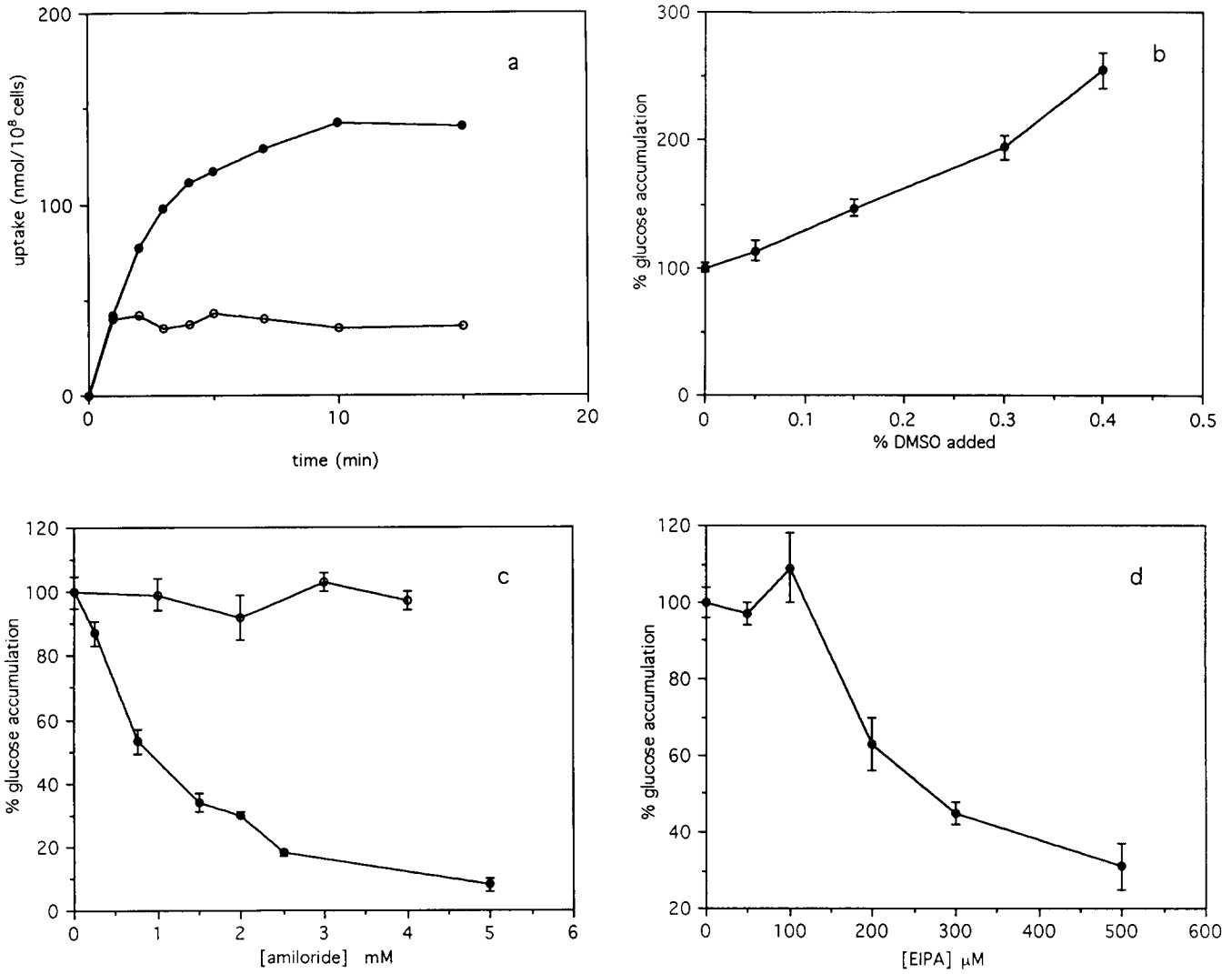

Fig. 5. Effect of amiloride and EIPA on glucose accumulation in *S. pombe*. (a) The accumulation of both glucose (•) and 2-DOG (○) was determined at several time points as described in Materials and Methods. (b) The effect of DMSO on glucose accumulation in *S. pombe*. Cells were incubated in the presence of increasing concentrations of DMSO. Glucose accumulation was determined as described in Methods. (c) Effect of amiloride on the accumulation of glucose and 2-DOG. Cells were incubated in the presence of increasing concentrations of amiloride, and then assayed for glucose (•) and 2-DOG (○) tracer as described in Materials and Methods. (d) Effect of EIPA on glucose accumulation. Cells were incubated in the presence of increasing concentrations of EIPA, and assayed for glucose tracer as described in Materials and Methods.

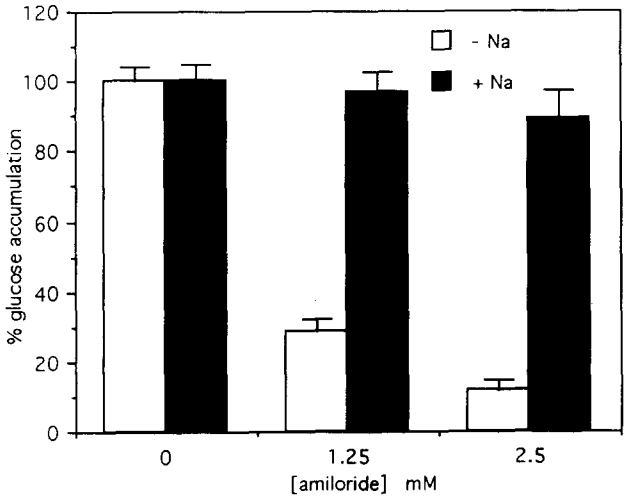

Fig. 6. The effect of sodium chloride on the inhibition of glucose accumulation by amiloride. Glucose accumulation was determined as described in Materials and Methods, in the presence (■) or absence (□) of 100 mM NaCl. Amiloride concentrations were as indicated in the figure.

analogues have been shown to be more selective in inhibition of the mammalian Na^+/Ca^{2+} and Na^+/H^+ exchangers [19], although this substitution can reduce specificity for other proteins, such as the Na^+ channel [19].

Amiloride and its analogs have been shown to inhibit a wide variety of cellular functions. These include various transport systems, protein kinases and second messenger systems [19]. No effects of these compounds on glycolysis have been reported. However, our results suggest that amiloride and EIPA both inhibit glycolytic flux in *S. pombe*, resulting in an inhibition of medium acidification. Future experiments will attempt to determine the precise site of action of these compounds.

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