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ENERGY-DEPENDENT UPTAKE OF CALCIUM BY THE YEAST *SCHIZOSACCHAROMYCES POMBE*

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Summary

1. In resting cells of the fission yeast *Schizosaccharomyces pombe*, the uptake of calcium is stimulated by the addition of 90 mM glucose in the presence as in the absence of respiration and inhibited by Antimycin A in the absence of exogenous carbon source. This uptake therefore requires fermentative or respiratory metabolic energy.

2. The calcium uptake by *S. pombe* exhibits saturation kinetics and high affinity for calcium. At external pH 4.5, the apparent K_m is 45 μM Ca^{2+}. 400 μM of other divalent cations exert competitive inhibitions of calcium uptake in the following order of affinities: $Sr^{2+} > Mn^{2+} > Co^{2+} > Mg^{2+}$. Inhibition by KCl is also observed but is of non-competitive type and requires high concentrations of the order of 40 mM.

3. At 30°C, the uptake rate of calcium is about 10-times higher at pH 8.25 than at pH 4.0. An extrusion of $^{45}Ca^{2+}$, the rate of which is estimated to be lower than one-fifth of the uptake, is observed in the presence of glucose when the external pH is acid.

4. At external pH 4.5, low concentrations of lanthanum chloride, ruthenium red and hexamine cobaltichloride are inhibitory for the uptake of calcium by the yeast cells.

5. In presence of Antimycin A, the uncouplers: NaN_3, dinitrophenol, and concentrations of carbonylcyanide *m*-chlorophenylhydrazone higher than 80 μM inhibit the calcium uptake by glycolysing cells. In the presence of glucose, the K^+ ionophore Dio-9 enhances severalfold the uptake of calcium even at 2°C.

6. It is concluded that *S. pombe* possess an active transport system for low concentrations of calcium. This transport seems to be dependent on an electric potential (negative inside) across the cellular membrane.

Introduction

In the yeast *Saccharomyces cerevisiae*, cellular uptakes of divalent cations such as Zn^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+}, Mg^{2+}, have been reported [1—6]. The transport of Ca^{2+} which is so extensively studied in mammalian cells and organelles is particularly poorly documented for yeast and fungi cells. The fission yeast *Schizosaccharomyces pombe*, does however, contain 20-times more calcium than magnesium. Most of the cellular calcium is released into the external medium after treatment by the divalent cations ionophore A23187 which also blocks nuclear division [7]. It was therefore concluded that a pool of calcium and magnesium is essential for cell division in *S. pombe* [7]. In the fungal coenocyte *Achlya*, the growth of which is strictly dependent on the presence of calcium, a passive uptake of Ca^{2+} and its binding to a glycoprotein have been demonstrated [8]. In *S. cerevisiae*, although it has been shown that the uptakes of Zn^{2+}, Co^{2+} and Ni^{2+} exhibit saturation kinetics [1—4], are stimulated by glucose [1], are balanced by the exit of K^+ [1], can be accompanied by the uptake of phosphate [3] and might be dependent of a non-mitochondrial adenosine triphosphatase [5,6], there is no clear evidence for a specific transport of Ca^{2+}. The same transport-system seems to be involved for all tested divalent cations including Ca^{2+} and Sr^{2+}, the affinities of which are lower than that of all other tested divalent cations [1]. The present communication indicates the existence of an energy-dependent uptake of calcium in the yeast *S. pombe*. This transport shows saturation kinetics and exhibits high preferential affinity for calcium. At external pH 4.5, it is inhibited by lanthanum chloride, ruthenium red and hexamine cobaltichloride. At the same pH, uncouplers and inhibitor studies lead to the conclusion that the uptake of low concentrations of calcium might be dependent of an electrical potential.

Materials and Methods

Yeast strain. S. pombe C0B5 is a strain which exhibits low respiratory activity on glucose but which is identical to the wild type when grown on glucose [9,10].

Culture conditions. The culture medium contained 36 g glycerol, 20 g yeast extract (Difco) and 1 g glucose in 1 l water. The medium was adjusted to pH 4.5 with HCl. Cultures were inoculated from a overnight preculture and grown in a 1-l Erlenmeyer flask containing 200 ml of growth medium at 30°C with vigorous shaking. After 24 h of culture, the exponentially growing cells were harvested by centrifugation and washed twice with cold sterile water. The pellets were kept on ice and used immediately.

Calcium uptake. The cells were suspended at the final concentration of $5 \cdot 10^7$ cells per ml in 20 mM piperazine-*N*,*N*'-bis (2-ethane-sulfonic) acid adjusted to pH 4.5 with HCl. Cellular suspensions of 1 ml were preincubated for 15 min at 30°C with continuous shaking in the presence of the indicated amounts of substrates, inhibitors and activators. 1 μM $^{45}CaCl_2$ (0.094 mCi per ml, 10^6 cpm) was then added and the cell suspension was further incubated for 15 min. The incubation was stopped by dilution of 500 μl samples of the cell suspension into 5 ml of cold water and by rapid filtration on Gelman filters

(0.8 μm pore size) prewashed with 20 mM $MgCl_2$. The cells were washed on the filter with 30 ml of 20 mM $MgCl_2$ to remove the calcium bound on the cellular surface.

Under these conditions, less than 5 pmol $^{45}Ca^{2+}$ was adsorbed per 10^8 cells, when the incubation was carried out at 2° C. Further washing with unlabelled $CaCl_2$ did not decrease the calcium adsorbed. The radioactivity of dried filters was measured in a toluene containing liquid with a scintillation spectrometer [11].

Calcium extrusion. After 15 min of incubation allowing the uptake of radioactive calcium as previously described, in the presence of 90 mM glucose, 15 μM Antimycin A and 1 μM $^{45}CaCl_2$, the cells were centrifuged and washed twice with 10 ml of 20 mM $MgCl_2$ and twice with 10 ml of cold sterile water. The cells were then resuspended in 20 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic) acid, pH 4.5 at the final concentration of $5 \cdot 10^7$ cells per ml and incubated again at 30° C in the presence of appropriate substrates or inhibitors. At indicated times, samples of 500 μl were filtered and their radioactivity measured as above.

Chemicals. $^{45}Ca^{2+}$ as $CaCl_2$ (0.94 mCi per ml containing 32 μg Ca^{2+} per ml) in aqueous solution was purchased from the Radiochemical Centre (Amersham). Antimycin A was from Boehringer; carbonylcyanide *m*-chlorophenylhydrazone from Calbiochem. Dio-9 was purchased from Koninklijke Nederlandsche Gist and Spiritus Fabriek, Delft, The Netherlands. When the chemicals were dissolved in ethanol, an equal volume of ethanol was added to the control.

Results

Metabolic energy-dependent calcium transport

The calcium uptake by *S. pombe* C0B5 was stimulated by the addition of 90 mM glucose, indicating that calcium uptake was energy-dependent. It was therefore of interest to determine the relative participation of glycolysis and respiration in the uptake of calcium. Fig. 1A shows the time course of calcium uptake in different metabolic conditions. In presence of 90 mM glucose, the uptake of calcium was linear for more than 15 min and reached a plateau of 75 pmol per 10^8 cells. The addition of 15 μM of Antimycin A, a respiratory inhibitor, to cells incubated with glucose decreased the rate of calcium uptake which however was linear for 20 min. In these conditions the incorporated calcium reached a maximum of 95 pmol per 10^8 cells, greater than that observed in the absence of Antimycin A. These kinetics were not modified by further addition of glucose suggesting that the energy supply was not limiting under these conditions. In the absence of glucose, when the respiration of endogenous substrates furnished the energy, the uptake was completely stopped within 35 min. Both glycolysis and respiration can thus supply the energy required for the transport of calcium.

Fig. 1B shows that in the presence of glucose and Antimycin A, an efflux of calcium could be observed in preloaded cells. This extrusion was linear with time and was not affected by the addition of unlabelled calcium to the incubation medium (data not shown). In the absence of glucose and in presence of

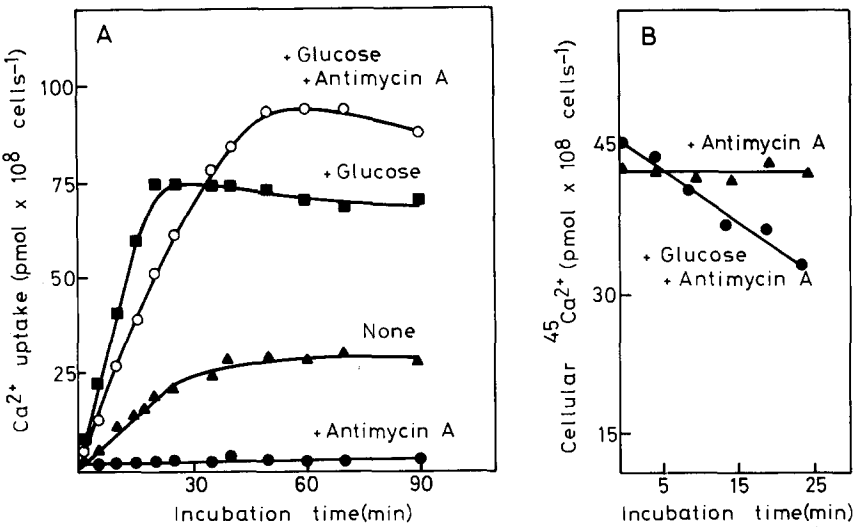


Fig. 1. (A) Effects of glucose and Antimycin A on the time course of the calcium uptake. Cells resuspended in 8 ml of buffer at pH 4.5 were preincubated in the presence of 90 mM glucose and 15 μM Antimycin A (○———○); glucose only, (■———■); Antimycin A only, (●———●); no substrate, (▲———▲). After a 15-min preincubation, 1 μM $^{45}CaCl_2$ was introduced into the suspension and samples were taken at the indicated times. B. Effects of glucose on the time course of extrusion of calcium. Cells loaded with $^{45}Ca^{2+}$ and washed as described in Materials and Methods were suspensed in buffer containing 15 μM Antimycin A, in presence, (●———●) or in absence, (▲———▲) of 90 mM glucose. Samples were taken at different time intervals.

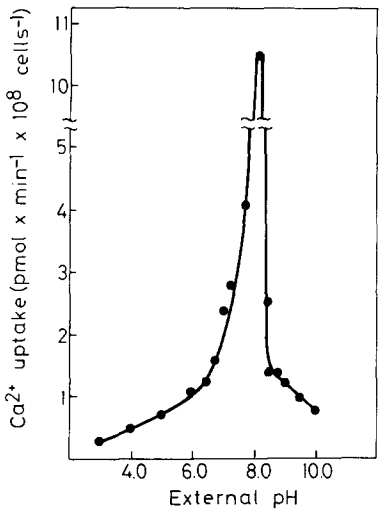
Antimycin A, no extrusion of calcium did occur suggesting that the efflux of calcium was also energy dependent.

Effect of pH

Fig. 2 shows that in presence of glucose and Antimycin A, the uptake of calcium increased sharply between external pH 7.2 and 8.2 Even though the absolute values of calcium uptake vary somewhat from one batch of cell to another, similar optima of pH between 7.2 and 8.2 were obtained in six distinct experiments. Although the calcium uptake was considerably increased under alcaline conditions, the following experiments were carried out at the external pH 4.5 which yields optimal growth of *S. pombe.*

Kinetics of the calcium uptake

Furhmann and Rothstein [1] reported the existence in *S. cerevisiae* of a divalent cation carrier, the affinity of which for calcium was estimated to be higher than 500 μM of Ca^{2+}. To determine the affinity of the *S. pombe* calcium transport system for its substrate, incorporated calcium was measured as a function of the external $CaCl_2$ concentration in presence of glucose and Antimycin A. It was calculated from the reciprocal double plot of Lineweaver-Burk that the apparent K_m and V were respectively 44 μM of Ca^{2+} and 0.066 nmol Ca^{2+} per min and per 10^8 cells (Fig. 3). In the presence of glucose but in absence of Antimycin A, the K_m was unchanged but V increased by a factor of 2.5 (data not shown). It is difficult to ascertain whether the small break seen at 20 μM $CaCl_2$ in the curve of Fig. 3 is of significance. However, at concentra-



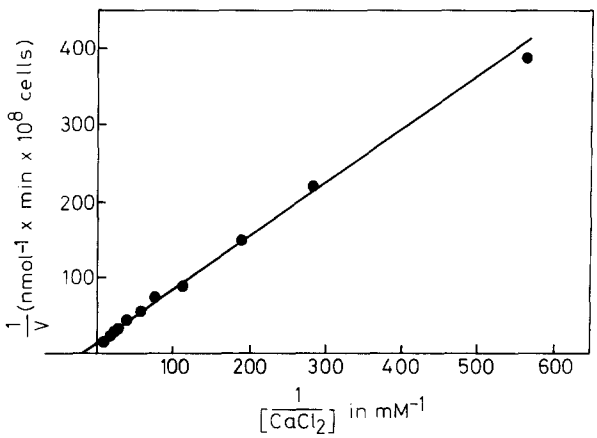


Fig. 2. Effect of external pH on the calcium uptake. $5 \cdot 10^7$ washed cells were suspended in 1 ml of 20 mM piperazine-*N*,*N*'-bis (2-ethane-sulfonic) acid (pH 3.0 to pH 8.5), 20 mM Tris (pH 8.5 to pH 9.5) or 20 mM glycylglycin (pH 9.5 and 10.0) adjusted to adequate pH with HCl or NaOH. The calcium uptake was then measured as previously described in the presence of 90 mM glucose and 15 μM Antimycin A.

Fig. 3. Saturation kinetics of calcium uptake in presence of glucose and Antimycin A. After preincubation of the washed cells in buffer at pH 4.5 in the presence of 90 mM glucose and 15 μM Antimycin A, increasing $CaCl_2$ concentrations were added. Calcium uptake was measured as previously described and the results were plotted as the reciprocal of the velocity of calcium uptake versus the reciprocal of the calcium concentrations.

tions higher than 150 μM $CaCl_2$, another Ca^{2+} transport system became apparent, the K_m of which was found to be higher than 300 μM (unpublished results).

To determine the specificity of the calcium transport system, the effects of 0.4 mM of the chloride salts of four divalent cations (Sr^{2+}, Mg^{2+}, Mn^{2+}, and Co^{2+}) were investigated at different $CaCl_2$ concentrations. Fig. 4 shows that the maximum velocity of calcium uptake was not significantly altered by the presence of other divalent cations. Competitive inhibition was observed since the apparent K_m of 44 μM for calcium uptake was increased to 130 μM by the presence of Sr^{2+}, to 106 μM by Mn^{2+}, to 92 μM by Co^{2+} and to 80 μM by Mg^{2+}. On the other hand, concentrations of K^+ as high as 40 mM were required to inhibit the uptake of calcium in a non-competitive way. These results show that the calcium transport exhibited significant affinity for other divalent cations. The uptake of K^+ seems to be mediated by a distinct transport system.

Whether the divalent cations exert a direct competitive effect or act indirectly by affecting the surface potential [12], cannot be specified from the present study.

Inhibition of calcium uptake by lanthanum chloride, ruthenium red and hexamine cobaltichloride

Three inhibitors of the active calcium uptake by rat liver mitochondria [13—16] were tested on intact *S. pombe* cells where respiration was blocked

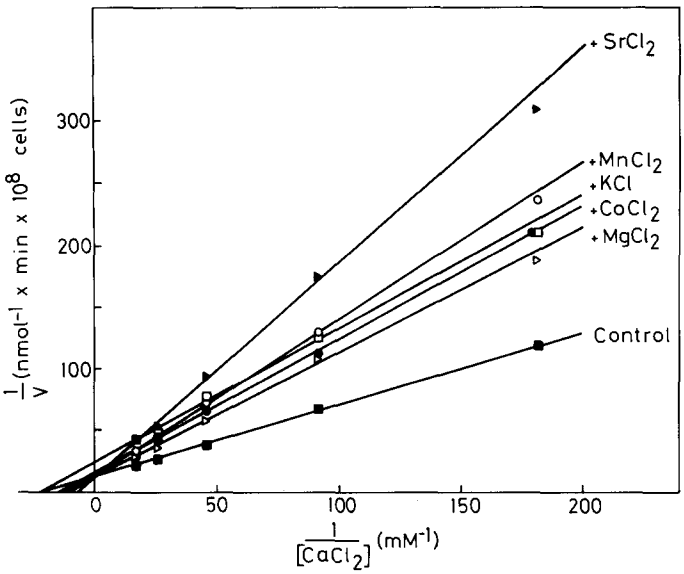


Fig. 4. Effect of K^+, Co^{2+}, Mg^{2+}, Mn^{2+} and Sr^{2+} on the kinetic constants of calcium uptake. Washed cells were preincubated in the presence of 90 mM glucose and 15 μM antimycin A. After 15 min of preincubation, five different $CaCl_2$ concentrations were added simultaneously to either, 0.4 mM $CoCl_2$, (●); $MgCl_2$, (▲); $MnCl_2$, (○); $SrCl_2$, (△) or 40 mM KCl, (□); each in a separate experiment.

with Antimycin A. In all cases, initial velocities of uptake were measured. At external pH 4.5, 28 μM lanthanum chloride reduced the uptake of calcium from 3.0 to 0.4 pmol per min and per 10^8 cells (Fig. 5A). Similar concentrations of hexamine cobaltichloride reduced the uptake from 2.3 to 0.6 pmol per min and per 10^8 cells (Fig. 5B). The calcium uptake was also decreased by low concentrations of ruthenium red (less than 10 μM) from 2.1 to 1.3 pmol per min and per 10^8 cells but was stimulated by a factor of two in the presence of 50 μM ruthenium red (Fig. 5C).

Effects of uncouplers on calcium uptake

In order to exclude any interference with mitochondrial oxidative phosphorylation, all experiments were carried out in the presence of glucose and

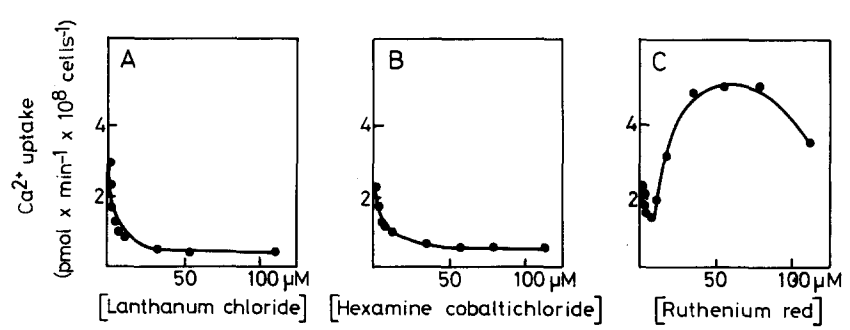


Fig. 5. Effect of lanthanum chloride, hexamine cobaltichloride and ruthenium red on calcium uptake. Cells were preincubated in the presence of 90 mM glucose and 15 μM Antimycin A and of increasing concentrations of $LaCl_3$, (A); hexamine cobaltichloride, (B) and ruthenium red (C).

Antimycin A. Figs. 6A and 6B show that at external pH 4.5, 20 μM of dinitrophenol and 200 μM NaN_3 inhibited strongly the uptake of calcium. On the other hand, low concentration (20 μM) of carbonylcyanide *m*-chlorophenylhydrazone stimulated the rate of calcium transport from 2.2 to 6.2 pmol per min and 10^8 cells (Fig. 6C). Actually, low concentrations of carbonylcyanide *m*-chlorophenylhydrazone prolong considerably the time course of calcium uptake (data not shown). This effect is not understood for the present time. However, the calcium uptake became almost completely inhibited by carbonylcyanide *m*-chlorophenylhydrazone concentrations higher than 80 μM.

Stimulation of calcium uptake by Dio-9

Inhibition of calcium uptake by uncouplers suggests that a membrane potential positive outside made, at least partly, of an electrogenic extrusion of protons across the cell membrane is required for the transport of calcium. It was therefore of interest to investigate how redistribution of H^+ and also K^+ across the cellular membrane influences the calcium uptake. For this purpose, an inhibitor of oxidative phosphorylation was used: Dio-9, an antibiotic of unknown structure [17]. It has been proposed that in rat liver mitochondria [18], low concentrations of Dio-9 exhibit ionophore effects by promoting a transmembrane K^+/H^+ exchange. In *S. pombe*, we have demonstrated that in the presence of glucose, Dio-9 induced a rapid cellular efflux of K^+ which was compensated by an influx of H^+ and probably Na^+ [19].

In the presence of glucose and Antimycin A, 20 μg Dio-9/ml stimulated rapidly and transiently the uptake of calcium in *S. pombe* (Fig. 7A). Under the same conditions, K^+ was extruded (Fig. 7B). In the same conditions but in absence of glucose, no stimulation by Dio-9 was observed and glucose could not be replaced by its analog 2-D-deoxyglucose (Fig. 7A).

In order to verify that the Dio-9 stimulated-calcium uptake was not the result of physical adsorption of calcium to the cell walls or membranes, the effect of Dio-9 on calcium uptake was investigated at 2°C. At this temperature, in the presence of Antimycin A and glucose, the uptake of calcium was extremely low, but the addition of Dio-9 (20 μg per ml) elicited disappearance of calcium from the incubation medium (Fig. 8). The uptake velocity was 2.7

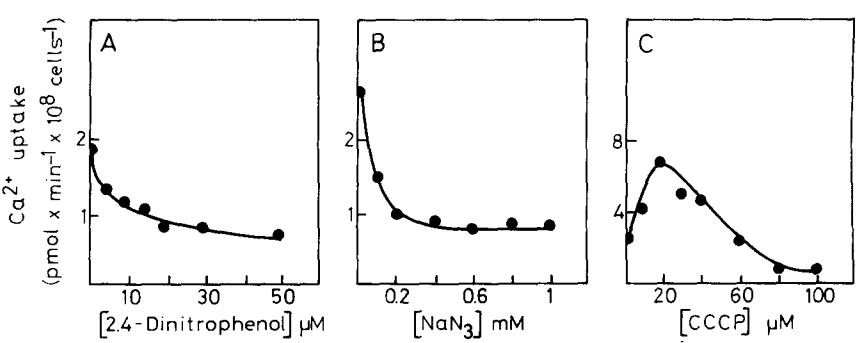


Fig. 6. Effect of uncouplers calcium uptake. Washed cells were preincubated in the presence of 90 mM glucose, 15 μM Antimycin A and increasing concentrations of NaN_3 (A); 2,4-dinitrophenol, (B) or carbonylcyanide *m*-chlorophenylhydrazone (CCCP), (C).

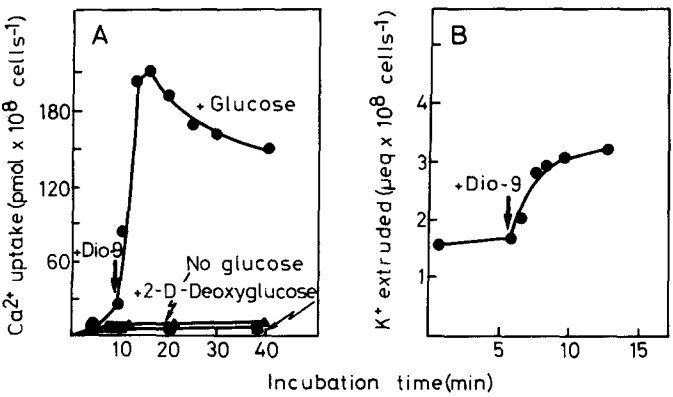


Fig. 7. Effect of Dio-9 on Ca^{2+} uptake (A) and K^+ extrusion (B). Cells were preincubated in the presence of 90 mM glucose and 15 μM Antimycin A (•). One experiment was carried out in presence of 90 mM of 2-D-deoxyglucose (■) instead of glucose. The control was with 15 μM Antimycin A but no glucose (▲). After the 15-min preincubation, $^{45}CaCl_2$ was introduced and 10 min later, 20 μg Dio-9 per ml were added. At indicated times, samples were taken. The uptake of calcium (A) was measured as described in Materials and Methods. For the measurement of K^+ extrusion (B), samples of 0.8 ml of cell suspension incubated in the presence of 90 mM glucose, 15 μM Antimycin A and where indicated 20 μg Dio-9 per ml, were immediately centrifuged for 15 s in a microfuge Eppendorf. Potassium contained in the supernatant was measured by flame photometry.

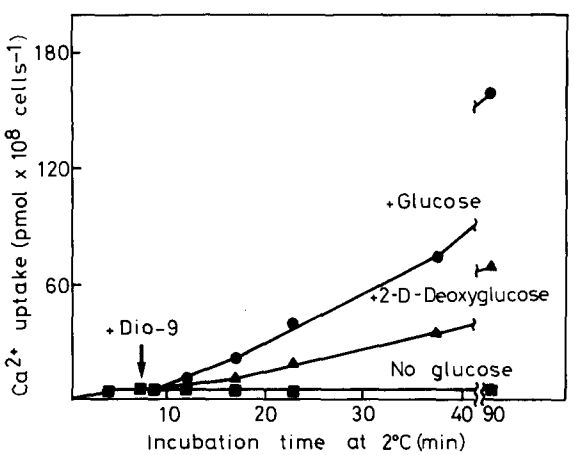


Fig. 8. Effect of Dio-9 on the time course of calcium uptake at 2°C. Experiment was carried out as described in Fig. 7, but the preincubation and incubation were carried out at 2°C instead of 30°C.

pmol per min and per 10^8 cells, i.e. three-fold slower than that at 30°C in the absence of Dio-9 but the uptake was still linear after 90 min, suggesting that Dio-9 was not merely inducing a cell surface calcium binding. Curiously, in the absence of glucose, Dio-9 did not elicit calcium uptake at 2°C and 2-D-deoxyglucose could be used instead of glucose.

Discussion

According to the following criteria: saturation kinetics, high affinity, specificity for the substrate, specific inhibitions, movement against a concentration gradient and requirement of metabolic energy, the uptake of calcium in

S. pombe cells can be characterized as an "active mediated transport". These six criteria will be considered one by one, below.

Saturation kinetics of calcium uptake are observed in *S. pombe*. At pH 4.5, in the presence of glucose and Antimycin A the K_m is 44 μM Ca^{2+} and V is 0.066 nmol Ca^{2+} per min and 10^8 cells. Fuhrmann and Rothstein describing the uptakes of divalent cations in *S. cerevisiae* [1], have suggested from ion competition studies that the apparent K_m of the carrier for calcium was higher than 500 μM. The system for calcium transport described here for *S. pombe* is therefore of high affinity compared to that previously described in *S. cerevisiae* [1]. Furthermore the inhibition of calcium uptake by KCl is less severe in *S. pombe* than that described by Fuhrmann and Rothstein for the uptake of divalent cations in *S. cerevisiae* [1]. It might be noted incidentaly that we have observed a high affinity calcium uptake in *S. cerevisiae* D261 and that another calcium uptake system for high concentrations exists in *S. pombe*. The latter uptake is independent of the presence of glucose and its apparent K_m is higher than 300 μM (personal data, not shown).

Among several other tested divalent cations, calcium is taken up by a high affinity transport-system of *S. pombe*. However, the specificity for the uptake of calcium is not absolute since it is competitively inhibited by other divalent cations. In this respect strontium is more effective than Mn^{2+}, Co^{2+} and Mg^{2+}. It is interesting to note that the apparent affinities of the calcium transport for various divalent cations follow the radius of the hydrated cations. It cannot be specified from this study whether the competitive ions only block the Ca^{2+}-binding site of a "carrier" or are really transported into the cell as proposed by Fuhrmann and Rothstein for the low affinity carrier of *S. cerevisiae* [1]. It must also be remembered that Theuvenet and Borst-Pauwels [12] have recently pointed out that apparent competitive uptake may occur between polyvalent cations and K^+ even when the polyvalent cations do not bind to translocation sites. More elaborated kinetic studies are obviously needed to resolve the possibilities raised above.

The respiration-driven calcium transport in rat liver mitochondria is inhibited by lanthanum chloride [13,14], ruthenium red [15] and hexamine cobaltichloride [16]. In this case, the inhibitors were thought to detect the presence of carbohydrate-components (ruthenium red and hexamine cobaltichloride) or phosphate groups ($LaCl_3$) at the calcium binding sites of the carrier. It has also been reported that in *S. cerevisiae*, ruthenium and lanthanum chloride, as well as Ca^{2+} increases the uptake of K^+ by stabilisation of the membrane [20]. The inhibitions by lanthanum, ruthenium red and hexamine cobaltichloride observed in *S. pombe* suggest that the cellular uptake of calcium involves chemical groups which ressemble those engaged in the active transport of calcium in rat liver mitochondria. The chemical nature of these groups is a matter of speculation at the present time and it cannot be determined whether they are directly involved in translocation or for instance, contribute to general screening by the above inhibitors, of the negative fixed charges on the surface of the yeast cell membrane, as recently proposed for polyvalent cations in *S. cerevisiae* [12]. It must be mentioned that although diphasic responses to ruthenium red and also to carbonylcyanide *m*-chlorophenylhydrazone are not explained at the present time, they are perfectly reproductible and obtained

under conditions where initial velocities are measured.

The cellular uptake of calcium in *S. pombe* is most likely carried out against a concentration gradient. Indeed the external concentration used in this work is 1 μM $CaCl_2$ and the intracellular calcium is estimated to be of order of 10 mM if one accept the content of $3 \cdot 10^{-14}$ g of soluble calcium per cell reported by Duffus and Paterson [7] and an average volume of 70 μm^3 per *S. pombe* cell [21]. The concentration gradient so calculated seems enormous and might be non-relevant since no information is available concerning the real nature and volume of the cellular compartment in which calcium is accumulated. A minimal internal concentration of calcium can however be estimated on the following bases: assuming that no endogenous calcium is present at the begining of the incubation and considering that the exogenous calcium is taken up in a cytosolic volume equal to the cell volume (70 μm^3), it can be calculated that an internal concentration of about 10 μM of calcium is build within 30 min. Uptake against a concentration gradient of calcium seems thus to operate in any case.

Transport of calcium by *S. pombe* is obviously linked to metabolic energy. The uptake is not carried out when the metabolism is reduced by low temperature and at 30°C either respiration or glycolysis are required for uptake of calcium. Our data clearly show that in the absence of exogenous substrates, the uptake is blocked by a respiratory inhibitor such as Antimycin A and that further addition of glucose restores the incorporation of calcium.

Our data concerning the effects of uncouplers and Dio-9 give additional information on the nature of the metabolic-dependent form of energy which is the driving force for calcium uptake. Studying the uptake of calcium by rat liver mitochondria, Rottenberg and Scarpa [22] concluded that calcium transport was an electrogenic process driven by a membrane potential. On the other hand, in microorganisms, according to Mitchell's chemiosmotic hypothesis [23,24], the accumulation of metabolites against a concentration gradient occurs in response to an electrical potential generated by an active extrusion of protons into the external medium. It is therefore important to know whether the calcium uptake by *S. pombe* is dependent or not on a membrane potential across the plasma membrane. Yeast cells actively extrude protons into the external medium [25]. Although the contribution of an electric component is not clear [26], an electrogenic extrusion of protons seems to occur across the cellular membrane [27] and in the absence of metabolic energy, an artificial pH gradient (acid outside) can drive the uptake of various metabolite against their concentration gradient [28]. In *S. pombe*, three distinct proton conducting agents: 2,4-dinitrophenol, sodium azide and concentrations of carbonylcyanide *m*-chlorophenylhydrazone higher than 80 μM inhibit the uptake of calcium. These results are in agreement with the previously reported inhibition by uncouplers of protons efflux or potassium and phosphate uptake in *S. cerevisiae* [29,30]. It is thus very likely that an electrogenic extrusion of protons is necessary for active cellular uptake of calcium in *S. pombe*. That an electric component does contribute to the driving force for the cellular uptake of calcium in *S. pombe* is also supported by the effects of Dio-9 which seems to induce a redistribution of cations on both sides of the cell barrier. The stimulatory effect of Dio-9 on calcium uptake confirms

previous suggestion that Dio-9 acts as an ionophore in rat liver mitochondria and intact cells of *S. pombe* [19].

In conclusion, we believe that because of its great potentialities of physiological and genetic manipulations, yeast furnishes a convenient model for the study of the cellular transport of calcium in eukariotic cells.

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