Efflux of Potassium Induced by Dio-9, a Plasma Membrane ATPase Inhibitor in the Yeast Schizosaccharomyces pombe*

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The active uptake of L-leucine is 50% inhibited by 10 µg of Dio-9/ml in glycolyzing cells of the yeast Schizosaccharomyces pombe in which the respiration has been inhibited by antimycin. Similar inhibition is observed in the nuclear respiratory-deficient mutant RD32 which lacks mitochondrial ATPase activity. Therefore the mitochondrial ATPase is not involved in the inhibition by Dio-9 of the cellular uptake of metabolites. On the other hand, the ATPase activities of purified plasma membrane fractions isolated from the wild type or the mutant RD32 were both 50% inhibited by 10 µg of Dio-9/ml.

Moreover, in the presence of glucose, Dio-9 induces an efflux of K⁺ simultaneous to an influx of H⁺ with a half-time for equilibration of 2 min. Uncouplers like carbonyl cyanide m-chlorophenylhydrazone increase the velocity of the potassium and proton movements induced by Dio-9 by reducing the half-time for equilibration to about 20 s. In the absence of glucose, Dio-9 does not induce cation movements unless uncouplers are present. The addition of CaCl₂ or NaCl decreases the H⁺ influx without modifying the Dio-9-induced K⁺ efflux, suggesting that Ca²⁺ and Na⁺ can substitute for H⁺ to balance the K⁺ efflux.

For increasing concentrations of Dio-9, the apparent stoichiometry of the K⁺ and H⁺ movements varies largely from negative to positive values finally tending toward 1.0 at saturating concentrations of the inhibitor. This variation reflects a dual action of Dio-9 on proton movements which can be discriminated by the effects of 80 mM external KCl which totally abolishes H⁺ influx at Dio-9 concentrations higher than 50 µg/ml but allows the expression of H⁺ efflux at concentrations lower than 10 µg of Dio-9/ml.

We conclude that Dio-9 elicits an electrogenic exit of K⁺ along the concentration gradient of this cation. The high electric membrane potential so generated must be balanced by the uptake of cations. Therefore in ATP-depleted cells, the ejection of K⁺ induced by Dio-9 can be observed only if a proton-conducting agent is added to the medium to balance the K⁺ efflux by an H⁺ influx. In the presence of glucose and Dio-9, protons or other cations such as Ca²⁺ or Na⁺ are taken up by the cell to neutralize the electric potential generated by the exit of K⁺. The Dio-9-induced K⁺ efflux is not second-

ary to the inhibition of the plasma membrane ATPase activity since it can be observed under conditions where the plasma membrane ATPase is either nonfunctional (absence of glucose) or not inhibited (low Dio-9 concentration).

The plasma membrane controls the cellular exit and entry of metabolites. According to Mitchell's chemiosmotic hypothesis, the active cellular transport in microorganisms is driven by an electrochemical potential difference generated across the plasma membrane by electrogenic extrusion of protons into the external medium (1). This "proton-motive" force would be produced either by oxidoreductions or by a plasma membrane ATPase. Earlier suggestions of an oxidation-reduction pump in yeast plasma membrane have not been confirmed (2), but electrogenic extrusion of protons has been reported (3), and it is clear that a pH gradient across the yeast cellular membrane can induce the uptake of metabolite (4-6). The plasma membranes of Saccharomyces cerevisiae (7-9) and Schizosaccharomyces pombe (10) contain an Mg²⁺-dependent adenosine triphosphatase and it has been shown that intracellular ATP is required for active cellular uptake in S. pombe (11). The involvement of a plasma membrane ATPase in active transport is thus an attractive hypothesis in yeast (12-14) as in Neurospora crassa (15, 16) and bacteria (17, 18), even though direct demonstration of this has not been furnished for yeast.

Chemical inhibitors provide useful tools to investigate the involvement of specific enzymes in complex biochemical functions, but specific inhibitors of the yeast plasma membrane ATPase have not been reported today. In this paper, we report that Dio-9, previously considered as a yeast mitochondrial ATPase inhibitor also inhibits the ATPase activity of purified plasma membrane fraction as well as the cellular uptake of L-leucine in the yeast S. pombe.

We also show that low Dio-9 concentrations elicit an exit of the cellular K⁺ down its concentration gradient. This electrogenic efflux can be balanced by the influx of protons or other cations such as Na⁺ or Ca²⁺. Higher concentrations of Dio-9 are required to inhibit the efflux of protons exhibited by glycolyzing cells which probably reflects the plasma membrane ATPase activity.

MATERIALS AND METHODS

Strains and Culture Conditions - Schizosaccharomyces pombe 972h⁻ was used as parental strain. The strain COB5 (10) and the
mitochondrial ATPase-deficient mutant RD32 (20) were previously described.

COB5 was grown in a medium containing 36 g of glycerol, 1 g of glucose, and 20 g of yeast extract (Difco)/liter. In these conditions, the respiration and other biochemical properties of this strain are identical with those of the wild strain (19). The nuclear respiratory-deficient mutant RD32 is unable to grow on nonfermentable carbon source and was grown in a medium containing 50 g of glucose and 20 g of yeast extract/liter. The media were brought to pH 4.5 with HCl. The cells were harvested in the exponential phase of growth.

Metabolite Uptake, Proton and Potassium Movements—The cells (50 × 10^8 in 1 ml) were incubated aerobically at 30° in 20 mM Tris-acetate, pH 4.5, for L-leucine uptake. For H^+ and K^+ movements, 5 × 10^8 cells were incubated at 25° in 5 ml of 2 mM Tris-citrate, pH 4.5. Proton movements were determined by measuring the extracellular pH with a Radiometer pH combination electrode (GR 3231C) connected to a Watanabe recorder (model MC3611). The scale was calibrated after each assay by addition of known amounts of NaOH. In all cases, the pH variations were lower than 0.05 pH unit.

The measurement of L-[U-14C]-leucine uptake was described in a previous article (11). For determinations of K^+ movements, aliquots of 500 μl of the cell suspension were centrifuged for 15 s in an Eppendorf microfuge. The supernatant was diluted five times with bidistilled water and the K^+ content was determined by flame spectrophotometry.

Isolation of Plasma Membranes—The following procedure for the isolation of plasma membranes from S. pombe was elaborated in our laboratory by Delhez (10). The cells (10 g) suspended in 20 ml of 50 mM Tris-acetate, pH 7.5, 250 mM sucrose, and 10 mM MgCl_2 were mixed with 10 g of glass beads (0.5 mm) and treated at maximal speed for 2 min with a refrigerated Braun homogenizer. The supernatant of a centrifugation for 10 min at 3,000 g for 20 min. Ten milliliters of the resulting supernatant were layered on 6 ml of sucrose at density 1.17 g/cm^3 and centrifuged at 100,000 x g for 10 min. The pellet was resuspended in 0.5 ml of 10 mM Tris-acetate, pH 7.5, 1 mM MgCl_2, and centrifuged at 130,000 x g for 1 h (Step 1).

The pellet was resuspended in 0.5 ml of 10 mM Tris-acetate, pH 7.5, and 1 mM MgCl_2, and mixed with 2.5 ml of sucrose at density 1.34 g/cm^3. On the top of this suspension, four sucrose bands of sucrose at densities 1.30, 1.25, 1.20, and 1.15 g/cm^3 were layered. The gradient was centrifuged at 100,000 x g for 15 h. The main purpose of this flotation discontinuous gradient was to separate the dense ribosome-containing fraction and plasma membranes migrated to the bottom of the tube. The pellet and the 2 ml located just above the pellet were collected and suspended in 15 ml of 10 mM Tris-acetate, pH 7.5, 1 mM MgCl_2, and centrifuged at 130,000 x g for 1 h (Step 2).

Adenosine Triphosphatase—The reaction mixture (1 ml) contained 25 mM Tris-acetate, pH 6.0, or 35 mM Tris-NaOH, pH 6.0, 6 mM MgCl_2, and 5 mM ATP. The reaction was started with about 50 μg of proteins and carried out at 30° for 8 min and stopped by 0.25 ml of 2.5 N perchloric acid. Inorganic phosphate was measured as described by Fullman and Penefsky (21).

ATP Content—The intracellular ATP was measured as described previously (11).

Protein Determination—The proteins were measured by the method of Lowry et al. with bovine serum albumin as standard (22).

Chemicals—Dio-9 was purchased from Koninklijke Nederlandsche Gin en Spiritus Fabriek, Delft, The Netherlands. The concentration of the stock solution of 2 to 5 mg/ml in ethanol was checked by the absorbance at 303 nm as reported by Guillory (23). Carboxylycine m-chlorophenylhydrazide was from Calbiochem and antimycin A from Boehringer. L-[U-14C]-Leucine (305 mCi/mmol) and **CuCl_2 (0.94 μCi/32 μg of Cu^++) were from the Radiodiagnostics Centre, Amersham.

RESULTS

Inhibition of L-Leucine Uptake by Dio-9 in Respiratory-Deficient Mutant—In a previous article (11), we have shown that in the absence of respiration blocked by antimycin, the cellular uptake of uridine in Schizosaccharomyces pombe COB5 was inhibited by Dio-9. Moreover, cellular growth was inhibited by Dio-9 both in glucose and in glycerol media, indicating that the mitochondrial ATPase was not the only site of action of the antibiotic.

The nonrespiring nuclear petite mutant S. pombe RD32 was used to further demonstrate a nonmitochondrial action of Dio-9. In this mutant, the specific activity of the mitochondrial Dio-9-sensitive ATPase, measured at pH 9.0, in the cell homogenate was less than 5% of that of the wild strain grown in the same conditions (20). Fig. 1 shows that the uptake of L-leucine in RD32 cells was 50% inhibited by 10 μg of Dio-9/ml of cell suspension. In this case, the virtually absent mitochondrial ATPase activity could not be the site of action of Dio-9, neither was it the production of glycolytic ATP which is not affected during the first minutes of incubation with Dio-9 (11).

Inhibition of Plasma Membrane ATPase by Dio-9—Purified plasma membranes of S. pombe contain an oligomycin-insensitive ATPase with maximum activity at pH 6.0 and virtually no activity at pH 9.0 (10). On the other hand, purified mitochondrial membranes exhibit maximum oligomycin-sensitive ATPase activity at pH 8.5 to 9.0. Both plasma membrane and mitochondrial ATPases are sensitive to Dio-9 (10). In Table 1, the specific activities of Dio-9-sensitive ATPase were measured both at pH 6.0 and at pH 9.0 in different steps of the purification of the plasma membranes of glycerol-grown COB5. The specific activity of Dio-9-sensitive pH 6.0 ATPase increased by a factor of 6.4 during purification (from 0.37 μmol of inorganic phosphate/min/mg of protein in the homogenate to 2.36 μmol of inorganic phosphate in the final plasma membrane fraction). The ratio of the activity of the pH 6.0 ATPase to that of the pH 9.0 ATPase increased from 0.7 in the homogenate to 56.0 in the purified plasma membrane fraction. The latter fraction was also enriched in mannans and did not contain mitochondrial cytochromes (10). It is clear that although the two purification steps largely decreased the mitochondrial ATPase, the enriched pH 6.0 ATPase activity was highly sensitive to Dio-9.

In the respiratory-deficient mutant RD32, the cell-free homogenate contained no detectable Dio-9-sensitive mitochondria.
Yeast Plasma Membrane ATPase and Dio-9-induced Efflux of K⁺

TABLE I

| Purification steps | Dio-9-sensitive ATPase |
|-------------------|-----------------------|
|                   | pH 6.0 | pH 9.0 | pH 6.0 | pH 9.0 |
| Homogenate        | 0.37   | 0.51   | 0.09   | <0.01  |
| Step 1            | 0.57   | 0.15   | 0.23   | <0.01  |
| Step 2            | 2.36   | 0.09   | 0.90   | <0.03  |

FIG. 2. Inhibition by Dio-9 of plasma membrane pH 6.0 ATPase activity in Schizosaccharomyces pombe COB5 and RD32. The two-step purification of the plasma membranes of both strains and the measurements of ATPase at pH 6.0 were described under "Materials and Methods." Proteins (130 and 250 μg) were used for Schizosaccharomyces pombe COB5 and S. pombe RD32, respectively.

FIG. 3. Effect of Dio-9 and carbonyl cyanide m-chlorophenylhydrazone (CCCP) on H⁺ and K⁺ movements across the cellular membrane of Schizosaccharomyces pombe COB5. Glycerol-grown cells of S. pombe COB5 were harvested in exponential phase of growth and transferred at the density of 10⁶ cells/ml (equivalent to about 1 mg of protein/ml) into a glass vial containing 5 ml of 2 mM Tris adjusted at pH 4.5 with citric acid. The cells were incubated for 2 min in presence of 10 μM antimycin. Proton and potassium movements were initiated at 25°C with 40 mM glucose. Dio-9 and carbonyl cyanide m-chlorophenylhydrazone, diluted in ethanol, were added at the final concentrations of 16 μg/ml and 50 μM, respectively. Proton and potassium movements were expressed in micromequivalents per ml of cell suspension.

FIG. 4. Time courses of potassium and proton movements induced by Dio-9. The addition of uncouplers such as carbonyl cyanide m-chlorophenylhydrazone (CCCP) prior to Dio-9 greatly increased the rate of both cation movements induced by the antibiotic (Fig. 3C) and reduced the half-time for equilibration across the cell membrane to about 20 s. Dio-9 has thus an action distinct from that of an uncoupler. In the absence of Dio-9, carbonyl cyanide m-chlorophenylhydrazone induced a much slower K⁺ efflux and H⁺ influx with a half-time for equilibration across the cell membrane of about 20 min (Fig. 3B). Similar K⁺ efflux induced by proton-conducting agents have already been described in S. cerevisiae (24). These results indicate that in the absence of uncouplers, the rate of potassium efflux is limited by the permeability of the cell membrane to protons. In other terms, Dio-9 does not make the cell membrane freely permeable to protons but seems to induce a leakage of K⁺ balanced by the uptake of protons.

Effect of Uncouplers—The addition of uncouplers such as carbonyl cyanide m-chlorophenylhydrazone (or 2,4-dinitrophenol) prior to Dio-9 greatly increased the rate of both cation movements induced by the antibiotic (Fig. 3C) and reduced the half-time for equilibration of H⁺ and K⁺ across the cell membrane to about 20 s. Dio-9 has thus an action distinct from that of an uncoupler. In the absence of Dio-9, carbonyl cyanide m-chlorophenylhydrazone induced a much slower K⁺ efflux and H⁺ influx with a half-time for equilibration across the cell membrane of about 20 min (Fig. 3B). Similar K⁺ efflux induced by proton-conducting agents have already been described in S. cerevisiae (24). These results indicate that in the absence of uncouplers, the rate of potassium efflux is limited by the permeability of the cell membrane to protons. In other terms, Dio-9 does not make the cell membrane freely permeable to protons but seems to induce a leakage of K⁺ balanced by the uptake of protons.

Specificity of Dio-9 toward Cat ions — It indeed the influx of
induced a K⁺ efflux of 0.32 pμeq x 10⁶ cells⁻¹ x 3 min⁻¹, similar to that observed in the absence of CaCl₂ (Fig. 3A) which was reduced to 0.12 pμeq x 10⁶ cells⁻¹ x 3 min⁻¹. However, the proton influx was reduced to 0.12 μeq x 10⁶ cells⁻¹ x 3 min⁻¹ (compared to 0.30 μeq x 10⁶ cells⁻¹ x 3 min⁻¹) and 0.085 μeq x 10⁶ cells⁻¹ x 3 min⁻¹ of ⁴⁰Ca⁺ was simultaneously taken up. These data indicate that Ca⁺ can substitute, at least partly, for the proton influx.

Fig. 5A shows that the addition of 40 mM NaCl after Dio-9 stopped immediately the inward movement of H⁺ without modification of the K⁺ efflux. Even though the movements of Na⁺ were not measured in that experiment it is quite likely that Na⁺ was taken up instead of H⁺ in exchange of the K⁺ efflux.

It can be concluded that the inward movement of cations elicited by Dio-9 is not restricted to H⁺, but that either monovalent cations such as Na⁺ or bivalent cations such as Ca⁺ can participate for balancing the extrusion of K⁺.

Effect of Glucose — A surprising aspect of this study was that the absence of glucose, Dio-9 had very little effect on the movements of K⁺ and H⁺ (Fig. 6A). However, the addition of a proton-conducting agent can substitute for glucose and under these conditions, Dio-9 induced a rapid exit of K⁺ and influx of H⁺ (Fig. 6B). This result can be understood if we assume that Dio-9 elicits an electrogenic efflux of K⁺ and that in the absence of glucose no inward movements of protons counterbalance the free positive charges (K⁺) leaving the cell. An electric membrane potential (negative inside) builds up quickly and stops the exit of K⁺. In the presence of a proton-conducting agent, a proton influx occurs in response to the K⁺ exit induced by the addition of Dio-9 and no intracellular efflux could be observed.

**Fig. 4.** Kinetic constants of the movements of K⁺ and H⁺ induced by Dio-9 in Schizosaccharomyces pombe COB5. The experimental conditions were the same as in Fig. 3A. The movements of K⁺ and H⁺ across the cellular membrane could be described by the following equations:

\[
\Delta[H^+] = \frac{A[H^{eq}] \times t}{T_{1/2} + t}
\]

\[
\Delta[K^+] = \frac{A[K^{eq}] \times t}{T_{1/2} + t}
\]

where \(\Delta[H^+]\) (or \(\Delta[K^+]\)) was the instantaneous decrease (or increase) of the concentration of H⁺ (or K⁺) in the external medium after Dio-9 addition. \(A[H^{eq}]\) (or \(A[K^{eq}]\)) was the difference between the concentration of H⁺ (or K⁺) in the external medium at zero time of Dio-9 addition and at equilibrium, \(t\) is the time after Dio-9 addition and \(T_{1/2}\) is the half-time required for equilibration. The reciprocal of the concentration of H⁺ (\(\Delta[H^{eq}]\)) or K⁺ (\(\Delta[K^{eq}]\)) was plotted versus the reciprocal of the time after Dio-9 addition. The half-time for equilibration of H⁺ (or K⁺) movements was determined by the intercept of the straight line on the abscissa. The variations of the concentration at equilibrium of H⁺ (\(\Delta[H^{eq}]\)) or K⁺ (\(\Delta[K^{eq}]\)) were determined by the intercept on the ordinate.

H⁺ is secondary to the Dio-9-induced efflux of K⁺, one might ask whether other cations could balance the electrogenic exit of K⁺. Fig. 5 illustrates the Dio-9-induced movements of H⁺ and K⁺ when Ca⁺ or Na⁺ were added in the external medium. The influence of the latter cations can be appreciated by comparison with Fig. 3A. We have already shown that Dio-9 stimulates Ca⁺ uptake (25). However, the low concentrations of ¹⁰⁶CaCl₂ used in this previous work did not permit the comparison with the simultaneous H⁺ and K⁺ movements. In Fig. 5B, 1 mM ¹⁰⁶CaCl₂ was added prior to Dio-9 and the antibiotic induced a K⁺ efflux of 0.32 μeq x 10⁶ cells⁻¹ x 3 min⁻¹, similar to that observed in the absence of CaCl₂ (Fig. 3A) which was 0.39 μeq x 10⁶ cells⁻¹ x 3 min⁻¹. However, the proton influx was reduced to 0.12 μeq x 10⁶ cells⁻¹ x 3 min⁻¹ (compared to 0.30 μeq x 10⁶ cells⁻¹ x 3 min⁻¹) and 0.085 μeq x 10⁶ cells⁻¹ x 3 min⁻¹ of ⁴⁰Ca⁺ was simultaneously taken up. These data indicate that Ca⁺ can substitute, at least partly, for the proton influx.

**Fig. 5.** Specificity of Dio-9 toward cation influx in Schizosaccharomyces pombe COB5. A, effect of NaCl on the movements of protons and potassium induced by Dio-9. The experiment was carried out as in Fig. 3A except that 40 mM NaCl was added 0.8 min after the addition of Dio-9. B, uptake of Ca⁺ and effects of CaCl₂ on the movements of protons and potassium induced by Dio-9. Glycerol-grown cells of S. pombe COB5 harvested in exponential phase of growth were transferred into 2 mM Tris-citrate buffer, pH 4.5, 10 μM antimycin, and 40 mM glucose. Then 1 mM ⁴⁰CaCl₂ (0.994 mCi/ml, 10⁶ cpm) was added 4 min after the addition of glucose and 2.5 min before the addition of 20 μM Dio-9. The movements of H⁺ and K⁺ were measured as described under "Materials and Methods." The measurements of calcium uptake are described elsewhere (25).
negative membrane potential opposes the K⁺ exit. In the presence of glucose, the exit of K⁺ is not electrolytic because protons can be taken up by the cell. However, even in the presence of glucose, the addition of a proton-conducting agent considerably increased the rate of H⁺ entry and K⁺ exit. It must therefore be concluded that glucose does not make the cell membrane permeable to protons. The possibility that protons are cotransported with glucose by a glucose/H⁺ symporter is rather unlikely since the analog 2-deoxyglucose, which is taken up by the cell but is an inhibitor of glycolysis, did not elicit appreciable H⁺ or K⁺ movements in the presence of Dio-9 (Fig. 6D). Cation movements were, however, observed in the presence of polysaccharides like raffinose or maltose (Fig. 6C) which are glycolyzed by S. pombe but not with nonmetabolized sugars such as galactose or 3-O-methylglucose (data not shown). It is therefore likely that catabolization of glucose, raffinose, or maltose brings the energy required for the apparent translocation of protons across the cell membrane.

**Relationship between K⁺ Exit and ATPase Inhibition**—Since in vitro the plasma membrane ATPase is sensitive to Dio-9, it was of interest to determine whether the exit of K⁺ was related to the inhibition of the ATPase. It must be mentioned that no effect of K⁺ has been observed so far on the S. pombe ATPase in vitro (10). Moreover, the ATP content of S. pombe COB5 incubated in the absence of substrates and in the presence of antimycin A and 30 mm glucose for 5 min before adding Dio-9. The movements of H⁺ and K⁺ were measured during the first 3 min after Dio-9 addition. In Fig. 6A, the K⁺ efflux and H⁺ influx are indicated by + values, H⁺ efflux is indicated by − values. B, the apparent stoichiometry of K⁺ efflux to H⁺ influx were calculated from the data of A.

**DISCUSSION**

The role of proton-translocating ATPase on the active transport of nutrients in bacteria has been widely discussed (17, 18). Coupling of metabolic energy and transport across the cell membrane is less documented in yeast, and the biological mechanisms for the generation of an electrochemical potential across the yeast plasma membrane are still obscure (3). In this communication, an attempt was made to determine whether the plasma membrane ATPase of the yeast Schizosaccharomyces pombe was playing a role in the active uptake of metabolites and which ions were involved in the generation of the membrane potential. We used an antibiotic of unknown
structure, Dio-9, which had been shown to inhibit oxidative phosphorylation and energy-dependent swelling in rat liver (23, 29). Mitchell and Moyle have suggested that in intact rat liver mitochondria, this antibiotic acts like gramicidin as an H\(^+\)K\(^+\) ionophore (27). On the other hand, rather high concentrations of Dio-9 inhibit the purified ATPases of yeast mitochondria (28, 29), bacteria (30, 31), chloroplasts (32), and Rhodospirillum rubrum (33).

The inhibition by Dio-9 of both L-leucine and plasma membrane ATPase could suggest that plasma membrane ATPase plays a role in the active uptake of nutrients in the yeast S. pombe. However Dio-9 is known to inhibit yeast mitochondrial ATPase (28, 29) and it is likely that the antibiotic affects the mitochondrial ATPase indirectly involved in active transport across the cell membrane. Specific mitochondrial ATPase inhibitors, however, such as venturicidin (11), oligomycin, and aurovertin (data not shown) do not inhibit the cellular uptake of L-leucine. Moreover, the uptake of L-leucine is 80% inhibited by 50 \(\mu\)g of Dio-9/ml of cell suspension in the wild type as well as in the respiratory-deficient mutant RD32 lacking the Dio-9-sensitive mitochondrial ATPase activity measured at pH 9.0. In this mutant, however, the purified plasma membrane possesses a high Dio-9-sensitive ATPase activity measured at pH 6.0 similar to that of the wild strain.

These results eliminate the possibility that Dio-9 acts on cellular transport by inhibiting the mitochondrial ATPase. They do not demonstrate however the involvement of the plasma membrane ATPase in active transport in yeast.

If Dio-9 inhibits a plasma membrane proton translocating ATPase in S. pombe, one could expect that the antibiotic stops the active extrusion of protons. This was indeed observed but at external pH 4.5, Dio-9 elicited also a rapid exit of K\(^+\) and an entry of H\(^+\), down the concentration gradient of both ions, with a half-time for equilibration across the cell membrane of about 2 min. These data raise the question whether the flux of K\(^+\) and H\(^+\) is related to the inhibition of the plasma membrane ATPase and what is the first event induced by Dio-9.

In ATP-depleted cells (no glucose and presence of antimycin to inhibit the respiration), Dio-9 does not induce significant movements of protons and potassium. However, if an uncoupler which makes the cell membrane freely permeable to H\(^+\) is added prior to the introduction of the incubation medium, a fast K\(^+\) exit is observed. In the presence of glucose, Dio-9 induces uptake of H\(^+\) simultaneously to K\(^+\) exit. However, even in the presence of glucose, uncouplers greatly increase the rate of K\(^+\) uptake induced by Dio-9. The half-time for equilibration of the cation across the cell membrane is reduced to 20 s instead of 2 min. These results can be interpreted as follows. (a) Dio-9 makes the cell membrane permeable to H\(^+\) and therefore induces leakage of K\(^+\) along its concentration gradient. (b) However, in ATP-depleted cells no active uptake occurs and in the absence of other ionic movements balancing K\(^+\) exit, the free positive charges (K\(^+\)) leaving the intracellular compartment generate an electric potential across the cell membrane preventing the exit of additional K\(^+\). (c) In these conditions, the addition of a proton-conducting agent which permits a H\(^+\) influx collapsing the electric potential enables Dio-9 to induce leakage of K\(^+\). In other words, the exit of K\(^+\) is balanced by the entry of H\(^+\). (d) In the presence of glucose, protons are apparently translocated into the cell in exchange of Dio-9-induced K\(^+\) exit. However since the rate of these movements is greatly accelerated by uncouplers, it must be concluded that the membrane is not permeable to H\(^+\).

Since Dio-9 is not active in the presence of 2-deoxyglucose which is transported but not glycolyzed, it is unlikely that the apparent proton uptake is associated with a sugar/H\(^+\) symporter. Since glycolyzed sugars such as maltose or raffinose substitute for glucose, the Dio-9-induced alkalization of the external medium is more likely dependent on glycolysis. A simple possibility in this respect is that in the presence of glucose and Dio-9, bicarbonate or other anions leave the cells together with K\(^+\) preventing the building of an electric potential and associating with protons in the external medium so that an apparent H\(^+\) influx is observed.

These results suggest strongly that the first event induced by Dio-9 is an electrogenic ejection of K\(^+\) balanced by the uptake of other cations such as H\(^+\), Ca\(^{2+}\), and possibly Na\(^+\). Is the potassium exit related to the inhibition of the plasma membrane ATPase? This hypothesis implies that the inhibition by Dio-9 of the plasma membrane ATPase would not only block the efflux of protons but also elicits a K\(^+\) exit. Although the plasma membrane ATPase of most eukaryotes is stimulated by K\(^+\), so far in S. pombe no effect of this cation has been observed in vitro. It cannot be excluded, however, that in intact cells, potassium activates the plasma membrane ATPase, and that the leakage of K\(^+\) occurs through the ATPase. On the other hand, we have shown that at low Dio-9 concentrations, in the presence of high KC\(_1\) concentrations in the incubation medium, no inhibitions of the efflux of protons can be observed. In the same conditions but at high Dio-9 concentrations, although no influx of H\(^+\) occurs, the efflux of H\(^+\) is completely stopped. These results suggest that (a) high extracellular concentrations of K\(^+\) prevent the efflux of K\(^+\); (b) low concentrations of Dio-9 do not inhibit the plasma membrane ATPase since when the exit of potassium is prevented by high external KCl concentrations the efflux of protons is not affected; (c) that high concentrations of Dio-9 do inhibit the plasma membrane ATPase, since no efflux of H\(^+\) is observed in the absence of K\(^+\) efflux.

In conclusion, the action of high concentrations of Dio-9 on the plasma membrane ATPase does not seem to be primary. The first movement induced by Dio-9 seems to be an electrogenic exit of K\(^+\) down the concentration gradient of this cation, balanced by the uptake of protons or other cations in order to neutralize the electric potential generated across the cell membrane by the exit of K\(^+\).

This work also points out the role of electric membrane potentials for the active uptake of cations such as Ca\(^{2+}\) and Na\(^+\) which can substitute for H\(^+\) to balance the Dio-9-induced K\(^+\) efflux. It must be mentioned, however, that even in the presence of Dio-9, glucose and energy are required for the uptake of these cations. In other words, the generation of an electric membrane potential such as that produced by the Dio-9-induced efflux of K\(^+\) is not sufficient to elicit the active uptake of the cations.

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