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ALL-OR-NONE INTERACTIONS OF INHIBITORS OF THE PLASMA MEMBRANE ATPase WITH *SACCHAROMYCES CEREVISIAE*

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The inhibitors of the yeast plasma membrane ATPase, Dio-9, miconazole, suloctidil, *N,N'*-dicyclohexycarbodiimide, triphenyltin and diethylstilbestrol provoke an all-or-none K^+ loss from the cells. Some of the K^+-depleted cells also show an increased permeability for the dye, Bromophenol blue, indicating that the barrier properties of these cells are drastically changed. Apart from this all-or-none process, a graded loss of K^+ is also observed. These observations warn against the use of the inhibitors in studies aimed at evaluating the role of the ATPase in the energy transduction of membrane transport processes of yeasts.

Introduction

The application of inhibitors of membrane ATPases in studies of the energization of transport processes is widely accepted in the literature. Among them, Dio-9 [1–7], miconazole [7], suloctidil [7], dicyclohexylcarbodiimide (DCCD) [1,7–14], diethylstilbestrol [1,8,11,12] and triphenyltin [3] have been used in studies of the energization of solute transport in yeast. Most of these compounds induce a K^+ efflux from metabolizing yeast cells [7,15]. This efflux is ascribed to the impairment of the membrane ATPase. However, if these compounds exert a cytolytic action as well, the provoked K^+ loss may equally well be interpreted as a secondary consequence of a loss in membrane integrity. This has been suggested to be the main cause of the Dio-9-induced amino acid efflux [16]. We have now examined whether the inhibitors concerned provoke a K^+ loss from yeast cells by destroying the permeability barrier of the cell, and whether all-or-none interactions of these inhibitors with yeast are involved.

Methods

Cells with a relatively homogeneous cell size distribution were obtained by suspending 40 g of pressed yeast, *S. cerevisiae*, Delft 2, in a 1000 ml cylinder. The cylinder was kept at 4°C for 1 day. Then the upper 100 ml was pipetted carefully without disturbing the yeast suspension. This upper layer contained only small-sized cells which were used in the experiments described. K^+ efflux from the yeast suspension at a cell density of $14 \cdot 10^6$ cells $\cdot$ ml^{-1} in 10 mM Mes/TEA buffer (pH 6.2) provided with 3% (w/v) glucose was determined by means of a K^+ glass electrode. Maximum K^+ efflux was determined after adding 125 μM CTAB to the yeast suspension. Accessibility of the cells to Tb^{3+} was determined by measuring the increase in fluorescence intensity due to

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; CTAB, cetyltrimethylammonium bromide; Mes, 4-morpholineethanesulfonic acid; TEA, triethanolamine.

interaction of Tb^{3+} with intracellular proteins [17] at the emission and excitation wavelengths 544 nm and 288 nm, respectively, with an Aminco SPF-500 fluorimeter. The fluorescence intensity found with cells boiled for 30 s was taken as 100 %, and the fluorescence found with intact cells as 0%. To 4.5 ml of the cell suspension was added 0.5 ml 9% NaCl. Then the cells were centrifuged and resuspended in 5 ml of water to which 0.25 mM $TbCl_3$ had been added. The fluorescence intensities were measured after 10 min.

For cell size determinations 0.1 ml samples were added to 10 ml 0.9% (w/v) NaCl. The cell sizes were determined by means of a Coulter counter model ZF equipped with a size-distribution analyzer model P 64. The threshold setting was 8%, the aperture was 32 and the attenuation was 0.25. The orifice had a diameter of 70 μm. Determination of the number of cells which were stainable with the dye bromophenol blue were carried out by centrifuging the 10 ml NaCl yeast suspension for 5 min at maximum speed in a Janetzki T32c centrifuge and carefully sucking off the supernatant. Then 0.2 ml of 0.5 mM Bromophenol blue in 7.5 mM Tris-succinate/2.5 mM $CaCl_2$ was added. The number of cells stained was counted by microscopic inspection.The potassium content of single cells was determined by means of the electron probe X ray-microanalysis technique [18]. 1.5-ml samples of yeast suspension were diluted with an equal volume of ice-cold 1.8% (w/v) NaCl or distilled water. Both methods gave essentially the same results. The cell suspension was subsequently sprayed through a glass capillary tube onto pure polished carbon plates, air-dried and stored under vacuum. For the determination of the X-ray spectra of the single cells and the analysis of these spectra (see Ref. 19).

Both Dio-9 and the yeast were gifts of Gist-Brocades, Delft. Miconazole and suloctidil were gifts of Professor A. Goffeau, Laboratoire d'Enzymologie, Université Catholique, Louvain-la-Neuve, Belgium. Triphenyltin, diethylstilbestrol, CTAB, DCCD and Bromophenol blue were from Merck-Schuckardt, Sigma, Aldrich and British Drug houses respectively. Dio-9, diethylstilbestrol and CTAB were dissolved in ethanol at 1 mg · ml^{-1}, 40 mM and 50 mM, respectively. DCCD, triphenyltin and suloctidil were dissolved in methanol at 100 mM, 10 mM and 20 mg · ml^{-1}, respectively. Miconazole was dissolved in dimethyl sulfoxide at 10 mg · ml^{-1}. Since Dio-9 is an impure compound of varying composition, we determined the extinction at 303 nm corrected for the contribution at 400 nm in order to allow comparison with other results reported. The extinction of 1 mg · ml^{-1} Dio-9 was 2.7.

Results

Fig. 1 shows that the loss of K^+ from metabolizing yeast induced by inhibitors of the membrane ATPase was accompanied by an increase in the number of cells which were stainable with the dye, Bromophenol blue. This dye does not enter intact cells of the strain of yeast used by us [21]. On the other hand, cells which are lysed either by boiling or by repeated freezing and thawing are readily stainable with this dye. Suloctidil- and triphenyl-

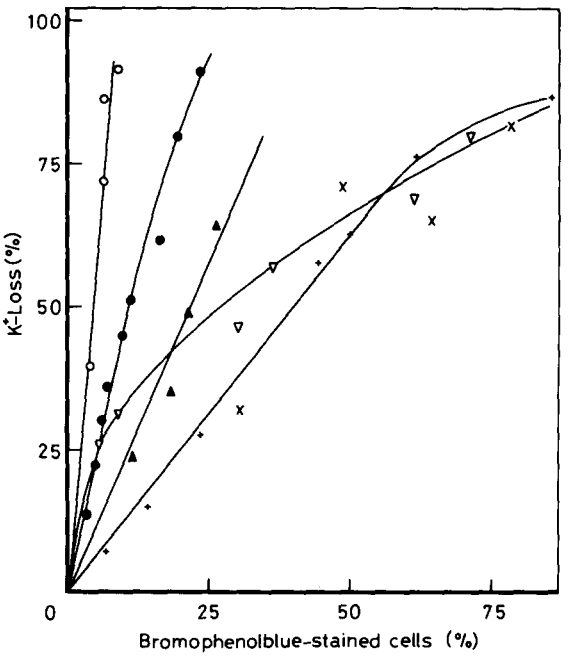

Fig. 1. Percentage of K^+ lost plotted against the percentage of cells stained by Bromophenol blue. ○, 25 μg · ml^{-1} Dio-9; ●, 25 μg · ml^{-1} miconazole; ▲, 0.2 mM dithylstilbestrol; ▽ 2.5 mM DCCD; +, 20 μg · ml^{-1} suloctidil; ×, 75 μM triphenyltin. The experiments with DCCD and diethylstilbestrol were carried out at 35°C, because of the low solubility of these compounds. The points plotted refer to data obtained at varying times of incubation with the inhibitors involved.

tin-induced K^+ efflux could be almost quantitatively accounted for by K^+ efflux from cells which had become leaky and which were accessible to Bromophenol blue. With DCCD, the first 30% of K^+ loss appeared to arise from still intact cells (see also Fig. 3B). The remainder of the K^+ came from cells which had lost their barrier properties. Approx. 50% of the K^+ loss found in the presence of 0.2 mM diethylstilbestrol could be accounted for by the increase in the number of permeabilized cells. The relative contribution of K^+ loss from permeabilized cells to the total K^+ loss was much smaller for miconazole and less still for Dio-9.

The K^+ loss appeared to be accompanied by a decrease in cell size. This was found with all compounds examined. We have now studied the time course of shrinkage induced by those inhibitors which gave rise to a K^+ loss which was accounted for by less than 50% of cell integrity. Fig. 2A shows the effect of miconazole upon the cell-size distribution. Two events took place. In the first place, the cells shrunk gradually and, secondly, a second population appeared which had a much smaller mean cell size than the original one. (This second population increased, whereas the population of larger cells decreased.) After addition of CTAB, all cells became small, so that only a single population remained. Similar results were obtained with diethylstilbestrol and DCCD (see Fig. 2B and 2C). For the effect of Dio-9 upon the cell size, see below.

The all-or-none shrinkage of the cells was also found on avoiding the increase in ionic strength caused by the dilution of the cell suspension in 0.9% NaCl. Addition of the inhibitors to a cell suspension in 50 mM buffer instead of the 10 mM buffer and measuring the cell-size distributions in the same 50 mM buffer showed that in this way also, an all-or-none shrinkage of the cells occurred in the presence of either triphenyltin, suloctidil, DCCD, miconazole or diethylstilbestrol (data not shown).

Fig. 3A shows that the loss of K^+ induced by miconazole was accompanied by an increase in the number of strongly shrunken cells. Apparently, the percentage of extremely shrunken cells was greater than the percentage of cells stainable with Bromophenol blue. Fig. 3A also shows the time-course of graded shrinkage of those cells which were not shrunken according to an all-or-none process.

Fig. 3B shows that with DCCD, incubated cells lost their K^+ initially only from still intact cells. The percentage of cells which were strongly shrunken increased sharply after 1 h (incubation with the inhibitor of membrane ATPase) in parallel with the number of cells stainable with Bromophenol blue. The percentage of Bromo-

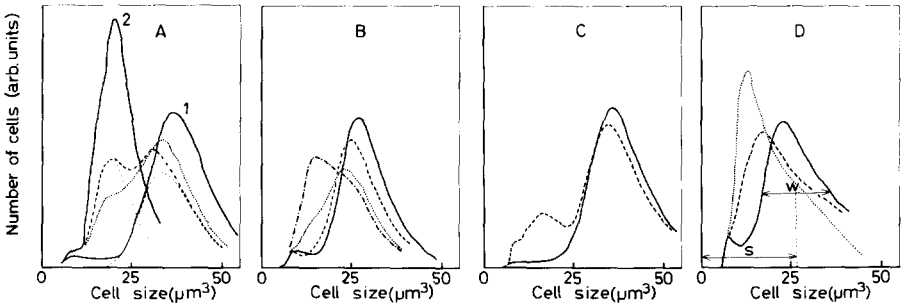

Fig. 2. Effect of inhibitors of membrane ATPase upon the cell-size distribution of the yeast cells. A. 25 $\mu g \cdot ml^{-1}$ miconazole: ——— 1, control; ——— 2, after addition of 125 μM CTAB; ····· after 7.5 min incubation with myconazole; - - - - - -, after 17 min. The distribution curve found after 17 min was resolved into two curves, one referring to the maximally shrunken cells, these cells having the same size distribution as the cells obtained after CTAB addition, and the other one referring to the cells which shrunk gradually (- - - - - -). B. 2.5 mM DCCD: ———, control; - - - - - -, after 60 min incubation with DCCD; ·····, after 120 min; and ·–·–·, after 145 min. C. 0.2 mM diethylstilbestrol: ———, control; - - - - - -, after 80 min incubation with diethylstilbestrol. D. 16 $\mu g \cdot ml^{-1}$ Dio-9: ———, control; - - - - - -, after 17 min incubation with Dio-9, ·····, after addition of 125 μM CTAB. The relative width of the distribution curve is $W' = W/S$. W is the width of the distribution curves at half the height of the curve. S is a measure for the mean cell size. We took the cell size referring to the middle of the line drawn at half-height of the distribution curve.

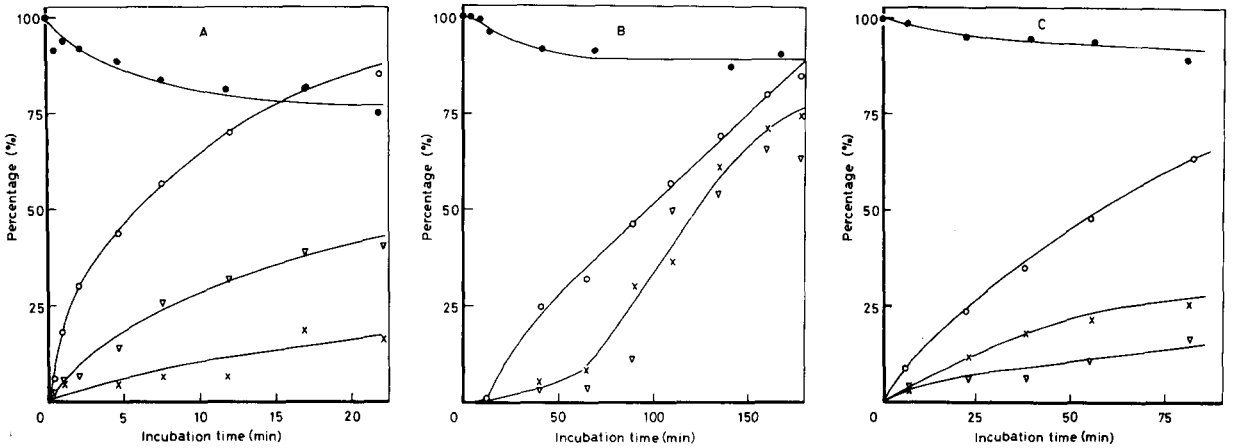

Fig. 3. Time-course of K^+ efflux induced by inhibitors of membrane ATPase, percentage of stainable cells, percentage of maximally shrunken cells and mean volume of those cells which shrunk via a graded process. ○, K^+ efflux expressed in percentage of the maximal K^+ loss obtained with 125 μM CTAB; ▽, percentage of cells shrunken maximally; ×, percentage of cells stainable with Bromophenol blue; ●, mean cell size of those cells which shrunk via a graded process expressed in percentages of the mean cell size before adding the inhibitor. A. 25 $\mu g \cdot ml^{-1}$ miconazole; B. 2.5 mM DCCD; C, 0.2 mM diethylstilbestrol.

phenol blue-stainable cells appeared almost equal to the percentage of cells shrunken via an all-or-none process. The rather slow rate of K^+ efflux could not be increased by increasing the DCCD concentration, because of the low solubility of DCCD.

Cells incubated with 0.2 mM diethylstilbestrol also showed a relatively small graded shrinkage smaller than that found with miconazole. Remarkably the percentage of cells stainable with Bromophenol blue was much greater than the percentage of cells which were strongly shrunken. This indicates that the dye interfered with the cell membrane, which membrane might be already weakened by the action of diethylstilbestrol. Similar results were found at 0.5 mM diethylstilbestrol.

With Dio-9, there arose in less than 10% of the cases examined two peaks in the cell size distribution patterns. Generally only one peak was found. However, the relative width of the distribution curves increased transiently during the shrinkage process, as shown in Fig. 2D. For the definition of the relative width, see the legend to Fig. 2D. When a cell-size population becomes less homogeneous, the relative width will increase. Therefore, the transient increase in the relative width found indicates that Dio-9 also gave rise to an all-or-none shrinkage of part of the cells, apart from a graded shrinkage.

We have now examined whether we could confirm that Dio-9 also provoked an all-or-none K^+ loss. The potassium content of single cells was determined by means of the X-ray microanalysis. The relative potassium content was expressed as the quotient of K and P (K/P). Fig. 4 shows that before addition of Dio-9 only a few cells had a very low K content, whereas the remainder of the cells belonged to a single K-rich population. During incubation with Dio-9, the number of K-poor cells increased and the number of cells with a relatively high K content decreased. Apparently some of the cells lost their K^+ via an all-or-none process. Typically, the percentage of K-depleted cells was always appreciably lower than that of cells stainable with Bromophenol blue (see Table I). Table I also shows that the mean K/P value of the K-rich cells decreased gradually during incubation of the cells with Dio-9. Thus apart from the all-or-none K^+ loss, a graded K^+ loss also occurred from the relatively K-rich cells.

The observation of a limited stainability of the K^+-depleted cells in the presence of Dio-9 does not rule out the possibility that Dio-9 still increased the permeability of the cells for small ions. In comparison to K^+, Bromophenol blue is a rather bulky molecule. We therefore examined

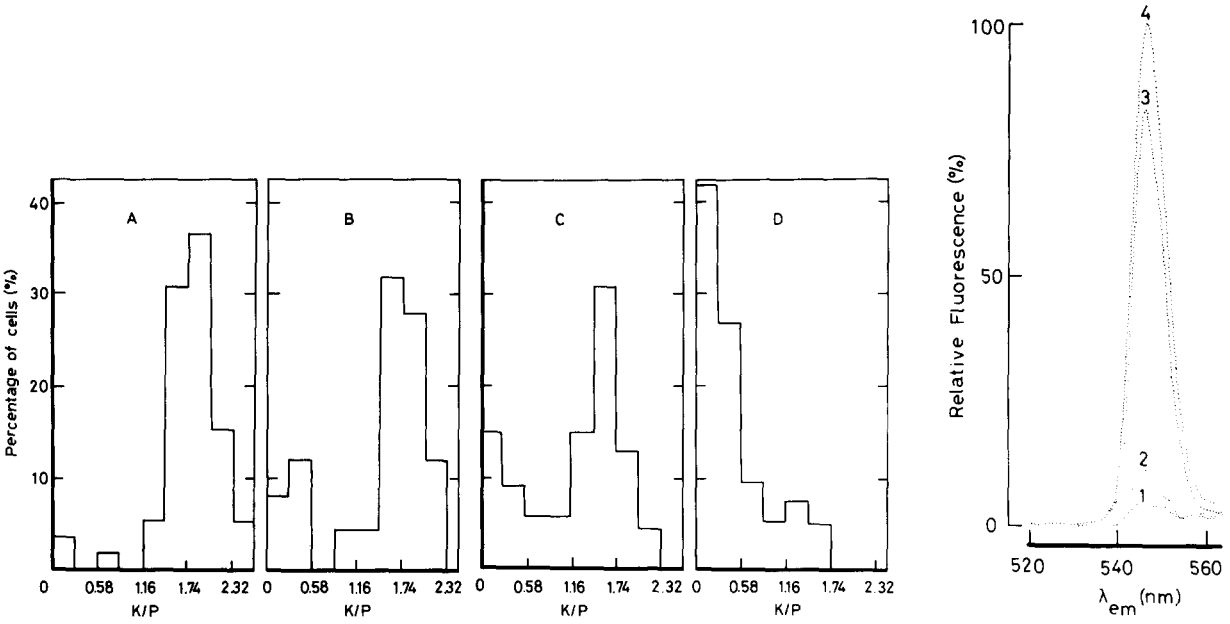

Fig. 4. Effect of Dio-9 upon the K distribution in metabolizing yeast cells determined by means of electron probe analysis of single cells. A, control cells; B, cells incubated for 6 min with 16 $\mu g \cdot ml^{-1}$ Dio-9. At 8 min the Dio-9 concentration was increased to 32 $\mu g \cdot ml^{-1}$. C and D, cells incubated for 10 min and 19 min, respectively.

Fig. 5. Enhancement of fluorescence of Tb^{3+} by binding to intracellular proteins made accessible by boiling the cells, treating them with CTAB or incubating them with Dio-9. Emission spectrum of Tb^{3+} at excitation wavelength 288 nm. 1, control cells; 2, cells incubated for 20 min with 25 $\mu g \cdot ml^{-1}$ Dio-9; 3, cells to which 125 μM CTAB was added and, 4, cells boiled for 30 s. The slightly lower fluorescence found with CTAB-treated cells was due to competition of Tb^{3+} with the small part of CTAB not removed by the washing procedure, for the binding sites on the intracellular proteins.

whether the much smaller Tb^{3+} cation might be able to enter part of those Dio-9 treated cells which were still impermeable to Bromophenol blue. Fig. 5 shows that the increase in fluorescence intensity of Tb^{3+} found after boiling the cells or after treating the cells with CTAB was much greater than the increase found after incubating the cells with Dio-9. In fact, the percentage increase in fluorescence intensity found with Dio-9 treated cells (7%) was equal to the percentage of cells stainable with Bromophenol blue, whereas approx. 70% of the K^+ was lost. Therefore those cells

TABLE I

NUMERICAL DATA REFERRING TO THE X-RAY MICROANALYSIS OF SINGLE CELLS TREATED WITH Dio-9

Data from Fig. 4. *BPB*: percentage of cells stained with Bromophenol blue. P_{LK} and P_{HK}: percentage of cells belonging to the low-K population and to the high-K population, respectively. K/P_{LK} and K/P_{HK} are the K/P ratios for the low-K and high-K cells, respectively, expressed as percentage of K/P_{HK} at zero time. K_{EPA} and K_{EL}: K loss calculated from the X-ray microanalysis data and from the K^+ determination with the K^+ electrode in the yeast suspension. S.E. are given.

Time (min)	*BPB* (%)	P_{LK} (%)	K/P_{LK} (%)	P_{HK} (%)	K/P_{HK} (%)	K_{EPA} (%)	K_{EL} (%)
0	1±1	5± 3	12±6	95± 3	100	0	0
6	2±1	20± 9	15±2	80± 9	94±3	18±3	22
10	4±1	30± 9	14±5	70± 9	86±3	35±3	35
19	13±2	80±10	10±6	20±10	66±5	73±9	63

which were still impermeable for Bromophenol blue, but which had lost the greater part of their K^+, were still not accessible to Tb^{3+}.

Discussion

Our results clearly show that the inhibitors of yeast plasma membrane ATPase examined all give rise to an all-or-none release of K^+ from metabolizing yeast cells. Apart from this all-or-none effect, most of them also induce a graded K^+ loss. All compounds investigated render part of the cells permeable to the anionic dye, Bromophenol blue. This shows that the integrity of the plasma membrane of part of the yeast cells is lost. This is supported by the fact that a distinct population of strongly shrunken cells is found after incubation of the cells with the inhibitor involved. These shrunken cells have the same size as is found for cells treated with the cationic detergent CTAB, which is known to destroy the barrier properties of the yeast cell completely [21].

With miconazole, the percentage of cells stainable with the dye Bromophenol blue is lower than the percentage of cells showing maximal shrinkage. This indicates that part of these shrunken cells are still impermeable for Bromophenol blue and thus have not lost the barrier properties of their membranes. On the other hand, with DCCD a close correspondence between the percentage of Bromophenol blue-stainable cells and maximally shrunken cells is observed. From the time-course of the increase in stainability of the cells and the appearance of maximally shrunken cells found with DCCD it may be concluded that the K^+ loss is primarily due to a graded process and that only after 1 h incubation is the all-or-none process also contributing appreciably to the K^+ loss. With miconazole, the contribution of the all-or-none process starts immediately, together with the graded process. This is also found with diethylstilbestrol. The K^+ efflux induced by Dio-9 also proceeds partly via an all-or-none process. The all-or-none process, however, is not due to a complete breakdown of the membrane. Only a small part of the cells becomes completely permeable to Bromophenol blue. Most cells are even inaccessible for the small cation Tb^{3+}. This does not support the supposition of Roon et al. [16] that the action of Dio-9 upon amino acid efflux is due mainly to a change in membrane permeability. On the other hand, it does not exclude the possibility that in the peculiar case dealt with by Roon et al. the permeability of the cells was greatly affected. In approx. 5% of the yeast samples examined by us, more than 50% of the K^+ lost during indubation with Dio-9 could be accounted for by the increase in the percentage of cells stained with Bromophenol blue (data not shown). The factors which are responsible for this incidentally greatly increased sensitivity of the yeast cell membrane for Dio-9 are not known to us. There is no indication that the age of the yeast contributes to the changes in sensitivity.

It may be considered that the changed in membrane properties provoked by the various inhibitors of membrane ATPase are indirect effects due to the inhibition of the ATPase. Inhibition of membrane ATPase will interfere with other energy-dependent processes, and may give rise to changes in the pH of the cytoplasm or to accumulation of toxic substances which are normally extruded from the cells. On the other hand, since some of the inhibitors lead to a complete breakdown of the cell membrane, whereas others hardly affect the general barrier properties of the membrane, the changes in membrane permeability found may be also additional effects of the inhibitors which are not directly related to the impairment of the membrane ATPase. In that case, the graded K^+ efflux may be due to the inhibition of the ATPase, whereas the all-or-none K^+ efflux may be caused by an interaction of the inhibitors with the cell membrane.

Dio-9 not only induces K^+ efflux, but also increases greatly the influx of Ca^{2+} both in *Schizosaccharomyces pombe* [15,22] and in *S. cerevisiae* (Boxman, A.W., unpublished data). In this respect Dio-9 resembles ethidium, which also induces K^+ efflux from *S. cerevisiae* and enhances Ca^{2+} uptake [23]. The ethidium provoked K^+ efflux proceeds also partially via an all-or-none process and partially via a graded process [19]. These findings may raise the question as to whether the enhanced Ca^{2+} uptake is restricted to only the K^+-poor cells or only the K^+-rich cells or, rather, occurs in both types of cell.

In the yeast *Rhodotorula gracilis* it is assumed

that triphenyltin inhibits specifically a K^+/H^+ antiport system [3]. Our results obtained with *S. cerevisiae* indicate that one should be very cautious with this suggestion unless it is ascertained that triphenyltin does not affect the membrane integrity of *R. gracilis*.

Our general conclusion is that conclusions based upon the action of inhibitors of yeast membrane ATPase about the way the energy transduction in yeast is regulated may be drawn only with great caution. In addition, as has been discussed in Ref. 24, some of the inhibitors applied are non-specific for plasma membrane ATPase and also act upon mitochondrial ATPase, which may further complicate the interpretation of results obtained with these inhibitors.

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