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Transient hyperpolarization of yeast by glucose and ethanol

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At pH 7, addition of glucose under anaerobic conditions to a suspension of the yeast *Saccharomyces cerevisiae* causes both a transient hyperpolarization and a transient net efflux of K^+ from the cells. Hyperpolarization shows a peak at about 3 min and a net K^+ efflux at 4–5 min. An additional transient hyperpolarization and net K^+ efflux are found after 60–80 and 100 min, respectively. Addition of 2-deoxyglucose instead of glucose does not lead to hyperpolarization of the cells or K^+ efflux. At low pH, neither transient hyperpolarization nor a transient K^+ efflux are found. With ethanol as substrate and applying aerobic conditions, both a transient hyperpolarization and a transient K^+ efflux are found at pH 7. The fluorescent probe 2-(dimethylaminostyryl)-1-ethylpyridinium appears to be useful for probing changes in the membrane potential of *S. cerevisiae*. It is hypothesized that the hyperpolarization of the cells is due to opening of K^+ channels in the plasma membrane. Accordingly, the hyperpolarization of the cells at pH 7 is almost completely abolished by 1.25 mM K^+, whereas the same amount of Na^+ does not reduce the hyperpolarization

Introduction

According to Kuschmitz and Hess [1] addition of glucose to a suspension of non-metabolizing *Saccharomyces cerevisiae* may lead to fluctuations in the membrane potential, as judged from transients in the fluorescence of rhodamine 6G with which the cells were preloaded. Until now, there have been no additional indications from literature for a transient hyperpolarization in yeast occurring after the onset of metabolism. This may be partly ascribed to the fact that most studies on the uptake of the lipophilic cationic membrane potential probe tetraphenylphosphonium (TPP) in *S. cerevisiae* are carried out after preincubating the cells for 60 min with glucose [2–4], thus giving no information about early changes in the membrane potential. In these studies, the equilibrium distribution of TPP between cell water and medium is determined. Neglecting eventual differences between activity coefficients of cell water and medium, the membrane potential is given by the Nernst potential

$$E_{TPP} = -\frac{RT}{F}\frac{TPP_{cell}}{TPP_{medium}} \quad (1)$$

TPP_{cell} is the concentration of free TPP in the cell water. Since part of the TPP inside the cell is bound to intracellular constituents, an appropriate

Abbreviations: TPP, tetraphenylphosphonium; DMP, 2-(dimethylaminostyryl)-1-ethylpyridinium; 2-DOG, 2-deoxy-D-glucose.

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correction should be applied when the total concentration of TPP is used instead of the concentration of free TPP. This correction is at least 19 mV and may even amount to 75 mV [4]. Studies in which the equilibrium distribution of TPP is examined only show that the steady-state membrane potential of metabolizing cells is more negative from interior to exterior than that of non-metabolizing cells. However, studies carried out with membrane potential probes other than TPP, in which the probe is added together with glucose [5,6] or shortly afterwards [7], do not provide any indication for the occurrence of a transient hyperpolarization of the cells.

We have to realize, however, that fluctuations in the membrane potential during the first minutes after the onset of metabolism may easily be overlooked. When the cells are transiently hyperpolarized for only a short period, uptake of TPP or any other probe may continue, provided that the cellular probe concentration is still below the expected final equilibrium concentration. Only when the initial accumulation of the probe involved is higher than would be expected at the subsequent partially depolarized state of the cells will a decrease in the cellular probe content occur after an eventual rapid initial hyperpolarization.

We have now examined whether conditions could be found under which a detectable transient uptake of TPP occurs. Furthermore we determined rates of TPP influx in the cells at varying times of preincubation of the cells with glucose. This method is more suited for the demonstration of changes in the membrane potential, especially when uptake of TPP in the cells is still relatively low, as compared to the uptake expected when TPP equilibrates between cell and medium and the membrane potential has reached a semi-steady state. We also exmained whether the fluorescent probe 2-(dimethylaminostyryl)-1-ethylpyridinium (DMP), which has been successfully applied for tracing changes in the membrane potential of mitochondria, trypanosomes and bacteria [8–10], can be applied to yeast cells as well.

Materials and Methods

10% (w/v) *S. cerevisiae* (Koningsgist, kindly provided by Gist-Brocades) was starved for 24 h in distilled water by aeration at room temperature. After exhaustion, the cells were washed twice with distilled water and suspended at a density of 2% (w/v) in buffer (45 mM Tris brought to pH 7.0 or pH 4.5 with succinic acid) at 25 °C. Glucose or ethanol was added to final concentrations of 5% (w/v) and 2% (v/v), respectively. Unless otherwise stated, the cell suspension was aerated in the presence of ethanol or bubbled through with N_2 in the presence of glucose.

Uptake of 0.36 μM [^{14}C]TPP was studied according to Ref. 4. Externally bound TPP was removed by washing the cells with an ice-cold solution of 20 mM $MgCl_2$.

DMP dissolved in 0.1% (v/v) methanol was added to a 2% (w/v) yeast suspension placed in a multi-purpose cuvet [11] at a final concentration of 1.4 μM. The fluorescence intensity was measured with an Aminco SPF 500 spectrofluorimeter at excitation and emission wavelengths of 470 and 565 nm, respectively. The cell suspension was continuously stirred and kept at 25 °C.

Net fluxes of K^+ were registered continuously by means of a valinomycin electrode placed in 30 ml 2% (w/v) yeast at 25 °C, bubbling through with N_2 or O_2, depending on the substrate added. A double-junction glass electrode (inner compartment filled with saturated KCl, outer compartment with 0.1 M $NaNO_3$) was used as a reference electrode.

All chemicals used were of analytical grade. ^{14}C-labeled TPP was purchased from Amersham International, Amersham, U.K. Trifluoperazine, 2,4-dinitrophenol, DMP and 2-deoxy-D-glucose (2-DOG) were from Sigma, St. Louis, MO, U.S.A., antimycin was obtained from Boehringer, Mannheim, F.R.G.

Results

On adding TPP and glucose simultaneously to the yeast suspension at pH 7, a rapid uptake of TPP into the cells occurred. This rapid uptake showed a lag period of approx. 1 min. Maximal uptake was reached after 10 min, and was followed by a slight though well-reproducible decrease in cellular TPP content, reaching a minimum at 16 min. A second maximum was found at approx. 60 min. Subsequently, the cellular TPP

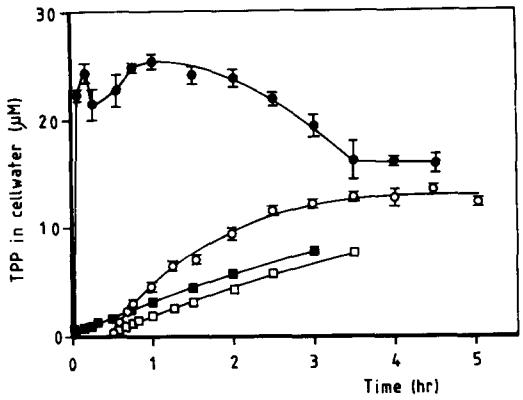

Fig. 1. Uptake of TPP in yeast at both pH 4.5 and 7.0 in the presence of glucose. Effect of preincubating the cells for 30 min with glucose. Closed symbols: glucose and TPP were added simultaneously to a 2% (w/v) suspension of non-metabolizing yeast cells. Open symbols: 5% (w/v) glucose was added 30 min prior to TPP. □, ○ pH 4.5 and 7.0, respectively. The height of the bars at pH 7 indicates the standard error of the mean. The number of determinations was eight. The experiments at pH 4.5 were carried out in duplicate.

concentration decreased gradually until an equilibrium distribution was reached.

When the cells were preincubated for 30 min with glucose before the addition of TPP, a much lower influx rate of TPP was found. In addition, no transients in TPP accumulation were observed. The final equilibrium distribution of TPP between cells and medium appeared to be of the same order of magnitude as that found when TPP and glucose were added simultaneously.

Contrary to what has been found at pH 7, at pH 4.5, simultaneous addition of glucose and TPP did not cause an extremely rapid uptake of TPP nor a transient decrease in the cellular TPP content. Furthermore, the influx rate of TPP did not significantly decrease on preincubating the cells for 30 min with glucose. Even after an incubation period as long as 210 min, no equilibrium distribution was reached for TPP between cells and medium.

In order to examine the time dependence of the transient hyperpolarization more accurately, we determined the influx rates of TPP as function of the time of preincubation. Fig. 2A shows that after a lag time of 1–1.5 min, TPP accumulation started. A maximum in the influx rate was reached after 3 min. Thereafter, the influx rate decreased again rapidly, showing a minimum at approx. 50 min. After that time, again, a slight increase in the influx rate was found for at least 20 min.

We also examined whether the initial transient hyperpolarization observed at pH 7.0 might be due to a transient hyperpolarization of the mitochondria caused by a small amount of O_2 still present in the yeast cell suspension. Exhaustion of this trace amount of oxygen may then again lead to a depolarization of the mitochondria and consequently to a decrease in TPP accumulation. However, impairment of mitochondrial respiration by 15 μM antimycin did not prevent the fluctuations in TPP uptake at pH 7. Furthermore on aerating the cell suspension, the same oscillations were found as when nitrogen was bubbled through the cell suspension (data not shown).

With ethanol as energy source and applying aerobic conditions, no transient uptake of TPP was found. At pH 7, the influx rate of TPP, however, was much higher when ethanol and TPP were added simultaneously than when the cells were preincubated for 30 min with ethanol before adding TPP (see Fig. 3). The differences in the rate of TPP uptake were less pronounced than those found in the presence of glucose.

In order to further confirm the transient hyperpolarization at about 3 min, we investigated whether the fluorescent compound DMP might be applied as a membrane potential for yeast cells and whether it would us enable to registrate

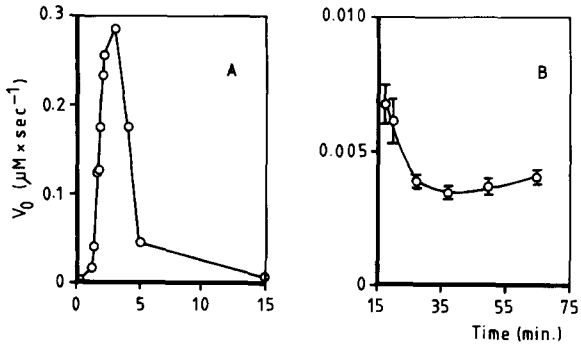

Fig. 2. Effect of addition of glucose upon the time dependence of the influx rate of TPP into yeast cells at pH 7 immediately after addition of 5% (w/v) glucose. The influx rates were determined by adding radioactive TPP at varying times of preincubation of the cells with glucose and taking the slopes of the uptake curves near zero time.

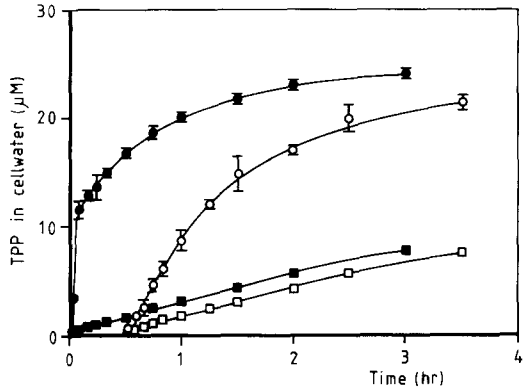

Fig. 3. Effect of 2% (v/v) ethanol upon TPP uptake at both pH 4.5 and 7. Effect of preincubation of the cells with ethanol upon TPP uptake. The experiments at both pH 7 and 4.5 were carried out in duplicate. For further details, see legend to Fig. 1.

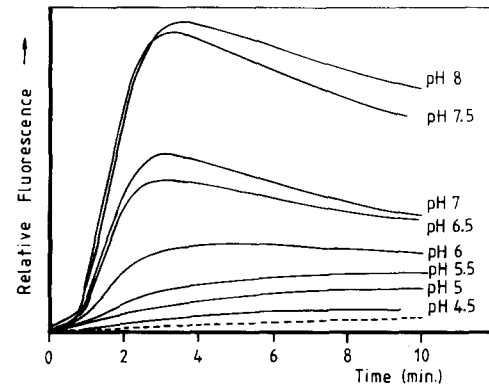

Fig. 5. Effect of addition of glucose to a suspension of 2% (w/v) non-metabolizing yeast upon the fluorescence intensity of DMP at varying pH. 1.4 μM DMP was added simultaneously with 5% (w/v) glucose at zero time. The effect of 15 mM 2-DOG upon non-metabolizing cells at pH 7 was studied in addition (dotted line).

changes in the membrane potential continuously. Fig. 4 shows that a hyperpolarizing agent like trifluoperazine [13,14] caused an increase in the relative fluorescence and that on depolarizing the cells with either 2,4-dinitrophenol [4] or 100 mM KCl [7], a decrease in the relative fluorescence was found. The directions of the changes in fluorescence intensity found on varying the membrane potential of the yeast were the same as has been found for other organisms [8–10].

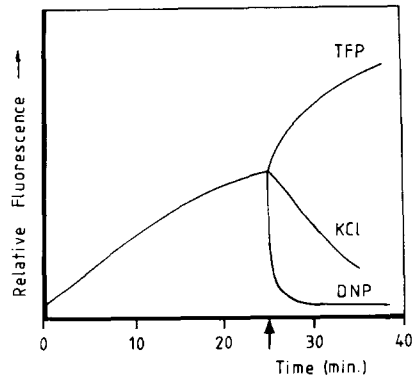

Fig. 4. Fluorescence intensity of DMP as a function of time. Effect of 0.050 mM trifluoperazine, 100 mM KCl and 0.050 mM 2,4-dinitrophenol added at 25 min after the simultaneous addition of glucose and DMP. 1.4 μM DMP was added to a 2% (w/v) suspension of non-metabolizing yeast together with 5% (w/v) glucose at pH 4.5. The fluorescence was continuously registered.

Fig. 5 shows that the probe DMP is well suited for detection of the initial transient hyperpolarization. The transient changes in the fluorescence intensity were more pronounced than those found for the uptake of TPP. The peak at pH 7 in the relative fluorescence almost coincided with the peak in the initial rate of TPP influx shown in Fig. 2A, whereas the peak in TPP uptake was found after about 10 min (see Fig. 1). At pH 5.5 and lower, no peak in the relative fluorescence was observed. The secondary peak in the membrane potential at pH 7 was also accompanied by an increase in fluorescence intensity showing up at about 80 min (data not shown). It was furthermore found that replacement of glucose at pH 7 by 2-DOG, which is only phosphorylated but not further metabolized [12], did not lead to a transient hyperpolarization.

Fig. 6 shows that at pH 7 and with glucose as substrate, after 1 min, a transient efflux of K^+ from the cells started. Maximal efflux of K^+ was found at 3.3 min, whereafter, K^+ was again taken up by the cells. At 40 min, a minimal K^+ concentration in the medium was reached, whereafter, a second period of K^+ efflux started with maximum efflux at about 100 min. After that time, the external K^+ concentration decreased again. Using ethanol as substrate and applying aerobic conditions, a rapid efflux of K^+ was found also after a 1 min lag period. Maximal K^+ efflux was found at

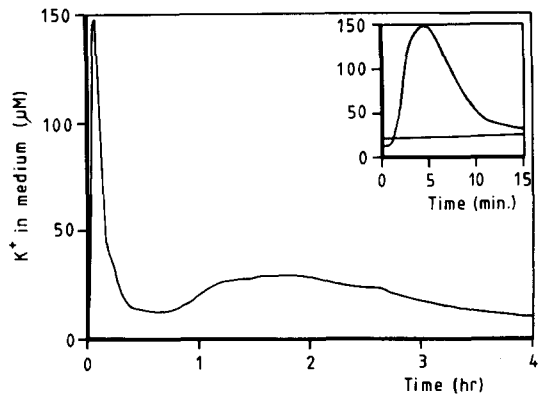

Fig. 6. Effect of glucose upon K^+ fluxes in yeast at pH 7. 5% (w/v) glucose was added to 2% (w/v) yeast suspension. The concentration of K^+ in the medium was continuously registered with a K^+-sensitive electrode. Inset: the time-course of the K^+ concentration in the medium immediately after the addition of glucose. Comparison with the effect of 15 mM 2-DOG added in the absence of glucose.

4 min (data not shown). When, instead of glucose, 2-DOG was added to the starved yeast cell suspension, no transient K^+ efflux was found. At pH 4.5, no net efflux of K^+ was observed after the addition of glucose or ethanol (data not shown).

Preliminary experiments showed that 1.25 mM K^+ reduced the fluorescence intensity of DMP 3 min after the addition of glucose by 88%, when glucose and K^+ were added simultaneously with the glucose to the yeast suspension at pH 7. On the other hand, the same amount of Na^+ did not decrease the fluorescence of DMP. This shows that the effect of K^+ is very specific. For a quantitative analysis of the effect of K^+ upon the membrane potential of the cells, we determined the influx rate of TPP at varying K^+ concentrations. When the influx rate of TPP depends in the same way upon the potential across the yeast cell membrane as the efflux, the influx rate will be given by

$$V_{TPP} = Q_{TPP} C_{TPP} \, e^{-FE/2RT} \tag{2}$$

F, R and T have their usual meaning. Q_{TPP} is a constant depending upon the permeability of the plasma membrane for TPP and the surface potentials at both the innerside of the cell membrane and the outerside (see Appendix). On increasing the concentration of K^+ in the medium, the gradient of K^+ across the cell membrane will decrease, and the cells will be depolarized. This depolarization may quantitatively depend upon the K^+ concentration in the medium according to the Goldman equation [15]

$$E = -\frac{RT}{F} \ln \frac{\Sigma P_i C_{i,\text{cell}} + \Sigma P_j C_{j,\text{medium}}}{\Sigma_i C_{i,\text{medium}} + \Sigma P_j C_{j,\text{cell}}}$$

$$= -\frac{RT}{F} \ln \frac{En}{Nu} \tag{3}$$

P is the permeability of the cell membrane for the ion concerned, the indices i and j refer to monovalent cations and anions, respectively. C represents the ion concentrations in cell water or medium. En and Nu are the enumerator and the numerator, respectively. The term Nu is responsible for the dependence of E upon the external K^+ concentration. Writing Nu as a function of $C_{K,\text{medium}}$ we get:

$$Nu = P_K C_{K,\text{medium}} + A \tag{4}$$

$A = \Sigma P_i C_{i,\text{medium}} + \Sigma P_j C_{j,\text{cell}}$, whereas index i is not identical with K. From Eqns. (2–4), it can be derived that

$$V_{TPP}^{-2} = B(A/P_K + C_{K,\text{medium}}) \tag{5}$$

with $B = Q_{TPP}^{-2} C_{TPP}^{-2} P_K En^{-1}$. Fig. 7 shows that a linear relationship exists between V_{TPP}^{-2} and $C_{K,\text{medium}}$ with $A/P_K = 40$ μM.

Discussion

For isolated mitochondria [8], trypanosomes [9] and bacteria [10], DMP has been shown to be a suitable probe for monitoring changes in the membrane potential. This study shows that this also applies to the yeast *S. cerevisiae*.

Apparently, at high external pH, a transient hyperpolarization is caused by addition of glucose to the cells. Part of the TPP accumulated into the cells during the first 3 min of incubating the cells with glucose is again extruded after that time. The decrease in TPP content is only transient and may easily be overlooked if one does not take enough data points within 30 min after the addition of

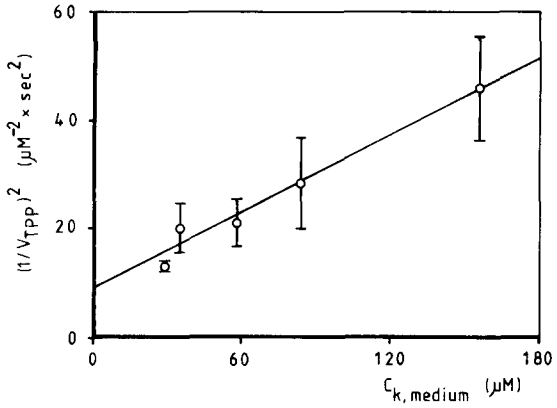

Fig. 7. Dependence of V_{TPP}^{-2} upon the K^+ concentration in the medium. Plot according to Eqn. 5. To a 2% (w/v) suspension of non-metabolizing cells, varying amounts of a KCl solution were added at zero time together with 5% (w/v) glucose and radioactively labelled TPP. The concentration of K^+ in the medium was registered continuously by means of a valinomycin electrode. N_2 was bubbled through the suspension. The concentrations of K^+ in the medium refer to the moment at which the influx rate of TPP was maximal. The rates of TPP uptake (V_{TPP}) are the maximal influx rates. The experiments were carried out in duplicate.

glucose to the cells. The changes in the influx rate of TPP during that period are far more pronounced.

Our studies furthermore show that with the fluorescent probe DMP, the transients in the membrane potential are easier to detect than by determining the uptake of radioactively labelled TPP, and that the peak in the fluorescence intensity is closer to the peak in the membrane potential, provided that the rate of TPP influx is a good relative measure for this potential. This indicates that DMP equilibrates more rapidly between cells and medium than TPP.

The transient hyperpolarization of the yeast cells is either directly or indirectly linked to the onset of metabolism. Addition of 2-DOG, which is only phosphorylated [12], and does not give rise to subsequent glycolysis, does not lead to this phenomenon. Furthermore, the effect is not restricted to the onset of glycolysis. Inducing respiration by the addition of ethanol under aerobic conditions also leads to a transient hyperpolarization. The hyperpolarization is accompanied by efflux of K^+ from the yeast cells. This may indicate that at the onset of metabolism, K^+ channels in the yeast cell [16] are opened. Because of the existence of a profound K^+ gradient between cells and medium [17], the cells will be highly hyperpolarized.

Apparently, the enlarged permeability of the cells for K^+ is a transient phenomenon. Remarkably, the transient hyperpolarization occurs only at high pH, but not at low pH. The absence of a hyperpolarization at low pH may be explained when the channels being opened also show proton conductivity. In that case, one would expect that at low pH, increased proton–K^+ exchange would be found. There are, however, no indications for increased K^+ efflux at pH 4.5. Another more likely possibility is that opening of the K^+ channels is impaired at low pH.

From the effect of K^+ upon the influx rate of TPP in hyperpolarized cells, it can be concluded that under these conditions, the membrane potential is highly sensitive to small amounts of K^+. Since A/P_K, see Eqn. 4, is small, the contribution of other cations from the medium (Tris cation and the small amounts of protons present at pH 7) do not contribute much to the resulting membrane potential. This also applies for permeating anions present inside the cell. We have also found that small amounts of Na^+ do not reduce the hyperpolarization. Therefore, the hyperpolarized cells behave by approximation as K^+ electrodes, which is in accordance with our view that during hyperpolarization, specific K^+-conducting channels are opened.

The membrane potential at maximal hyperpolarization may be calculated by means of Eqn. 3. Taking into account that $A/P_K = 40$ μM, a value of -209 mV is obtained. In this calculation, it is also assumed that $En = P_K C_{K,cell}$, with $C_{K,cell} = 250$ mM.

The membrane potential at maximal hyperpolarization can also be calculated in another way. 18 min after the addition of glucose at pH 7 (see Fig. 2), TPP uptake shows a minimum. Then Eqn. 1 may be applied, resulting in an apparent membrane potential amounting to -125 ± 3 mV. On accounting for binding of TPP to intracellular constituents, E becomes -111 ± 3 to -51 ± 3 mV, depending upon the way the binding of TPP is determined [4]. The influx rate of TPP is 0.0068 $\pm$ 0.0006 $\mu M \cdot s^{-1}$, whereas at maximal hyper-

polarization, the influx rate is $0.25 \pm 0.03\ \mu M \cdot s^{-1}$. The ratio of the fluxes is 48 ± 8. According to Eqn. 2, the membrane potential at maximal hyperpolarization would be 184 ± 9 mV more negative than at 18 min. In this way, a value between -295 and -235 mV is obtained for the membrane potential at maximal hyperpolarization, appreciably higher than the -209 mV calculated by means of Eqn. 3.

At this stage, we can only guess about the possible explanation for this discrepancy. In our calculation of the membrane potential at maximal hyperpolarization by means of Eqn. 3, we have made the approximation that *En* only consists of a K^+ concentration-dependent term. The contribution of the ATP-dependent proton pump of the yeast cell [18] is not taken into account. A second factor which may contribute to the observed discrepancy is that the surface potential becomes more negative on opening the K^+ channels. Finally, we cannot completely rule out the possibility that the permeability of the cell membrane for TPP may be transiently increased.

Normally, the negative membrane potential of metabolizing yeast cells is much less than the K^+ equilibrium potential [4]. This indicates that under these conditions, the permeability of the cells for K^+ is relatively low. Furthermore in non-metabolizing cells, the membrane potential is less negative still, indicating that an appreciable part of the membrane potential is due to the electrogenic proton uniporter [18].

Both TPP uptake at pH 7 and DMP fluorescence show a second peak 60–80 min after addition of glucose to the yeast suspension, which is accompanied by a transient decrease in the net rate of K^+ uptake. This may indicate that again, part of the K^+ channels are opened. The slight subsequent decrease in the cellular TPP content or DMP fluorescence may be due to a decrease in metabolism caused by gradual exhaustion of glucose in the medium.

Possibly, opening of the K^+ channels is triggered by a transient increase in the cAMP content of the cells [19]. Experiments aimed to support this notion further have now started.

Besides indirect indications from the changes in fluorescence of intracellular rhodamine 6G [1], until now, no indications have been found for a transient hyperpolarization of *S. cerevisiae* by glucose at pH 6–8 [5–7,20]. This, however, does not rule out the possibility that in these cases, a transient hyperpolarization still has occurred. The overshoot of the membrane potential may have been much briefer, and may have remained unobserved on applying relatively slowly penetrating probes for the membrane potential. A transient hyperpolarization can be detected in such a case by determining initial rates of influx at varying times of incubation of the cells with metabolizable substrate. As a matter of fact, the transient hyperpolarization provoked by ethanol under aerobic conditions at pH 7 could only be detected by us on comparing the initial rates of uptake of TPP added at both zero time and 30 min after the addition of ethanol.

Appendix

The meaning of the 'constant' Q_{TPP} in Eqn. 2 can be seen as follows. The membrane potential across the yeast membrane (E) is a function of the surface potentials at the outerside, E_o, and the innerside, E_i, and the potential between the two sides of the cell membrane, E_m

$$E = E_o + E_m - E_i \tag{A-1}$$

The influx rate of TPP is given by Eqn. 2A, see also Ref. 21

$$V_{TPP} = P_{TPP} C_{TPP,medium}\, e^{-FE_o/RT}\, e^{-FE_m/2RT} \tag{A-2}$$

On eliminating E_m from Eqn. 2A, we get Eqn. 2, with

$$Q_{TPP} = P_{TPP}\, e^{-F(E_o+E_i)/2RT} \tag{A-3}$$

Acknowledgements

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