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THE SITE OF ACTION OF 2,4-DINITROPHENOL AND SALICYLIC ACID UPON THE UNCOUPLER-INDUCED K^+ EFFLUX FROM NON-METABOLIZING YEAST

J.A. HOEBERICHTS, TH.J.M. HULSEBOS, P.M.G.F. VAN WEZENBEEK and G.W.F.H. BORST-PAUWELS

Laboratory of Chemical Cytology, Faculty of Science, Uncoupler action; (Yeast) Toernooiveld, Nijmegen (The Netherlands)

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Summary

Stimulation of K^+ efflux from non-metabolizing yeast cells by 2,4-dinitrophenol or by salicylic acid occurs only after accumulation of the compounds into the cells, indicating that the site of action of the uncouplers is inside the cells. A correlation is found between the partition ratio of the lipophilic cation dibenzyldimethylammonium between cells and medium and the rate of K^+ efflux.

Introduction

The rate of K^+ efflux from non-metabolizing yeast cells at low pH is increased considerably on adding 2,4-dinitrophenol [1]. This K^+ efflux is accompanied by a raise in the medium pH [2,3]. The latter might be due to an increase in the permeability of the yeast plasma membrane to protons. The inward flux of protons will lead to depolarisation of the yeast membrane giving rise to an increase in the rate of K^+ efflux. However, as pointed out by Mitchell and Moyle [4], an increase in medium pH will be found also when a steady influx of the undissociated dinitrophenol occurs. This will be true when the anionic form of dinitrophenol (dinitrophenolate) after being accumulated into the cells crosses the cell membrane in the outward direction in response to the electrochemical potential. This potential is composed of a contribution of the membrane potential, which is probably negative from inside to outside [5,6] and the concentration gradient of dinitrophenolate, which is positive, because at low medium pH the cell pH is much higher than the medium pH [7]. Then a concentration gradient of the undissociated acid between medium and cells

will be maintained until the pH of the cells equals that of the medium. Depolarisation will occur as well due to diffusion of the dinitrophenolate through the yeast cell membrane.

Evidence will be given that both 2,4-dinitrophenol and salicylic acid stimulate K^+ efflux from non-metabolizing yeast cells only after accumulation into the yeast cells. The increase in the rate of K^+ efflux is accompanied by a decrease in the apparent membrane potential determined via the distribution of the lipophilic quaternary ammonium-base dibenzyldimethylammonium.

Materials and Methods

Yeast, *Saccharomyces cerevisiae*, Delft II, loaded with $^{86}Rb^+$ is prepared by incubating the yeast aerobically at 2% (w/v) in 45 mM Tris-citrate buffer of pH 4.5, provided with 1% glucose (w/v) and carrier free $^{86}RbCl$, for 20 h at about 22° C. The glucose is added in order to ensure the uptake of $^{86}Rb^+$. Then the yeast is washed three times with ice-cold buffer and the cells are resuspended in buffer of 0° C and pH 4.5 without glucose, while nitrogen is bubbled through the suspension. Efflux of $^{86}Rb^+$ from these cells to buffer of pH 4.5 is studied by taking 5-ml samples which are centrifuged at appropriate times. The supernatants are assayed for radioactivity with a G.M. tube.

Efflux of K^+ from non-metabolizing cells suspended in buffer of pH 4.5 without glucose at 25° C is examined by determining by means of flame photometry the concentration of K^+ in the supernatant obtained by centrifuging the yeast suspension at appropriate times.

Uptake of ^{14}C-labelled 2,4-dinitrophenol and salicylic acid, both from the Radiochemical Centre, Amersham, England, is determined according to Borst-Pauwels [8]. ^{14}C-labelled dibenzyldimethylammoniumchloride has been prepared according to Lombardi et al. [9]. The 'equilibrium' distribution of the cation added at a final concentration of 10^{-6} M between cells and medium is determined according to Hoeberichts and Borst-Pauwels [5]. Cell pH's are determined according to Borst-Pauwels and Dobbelmann [10].

The ATP content of the yeast cells is determined with the luciferine-luciferase method. The cells are extracted with $HClO_4$. The cell extract is brought at pH 7 with KOH.

Results

It has been shown in orienting experiments (see also Fig. 2) that the rate of dinitrophenol-stimulated K^+ efflux from non-metabolizing cells at 25° C is already maximal within 1 min. Also the accumulation of dinitrophenol into the cells appears to be completed within that time. As Fig. 1 shows, the uptake of dinitrophenol at 0° C proceeds much slower. The dinitrophenol stimulated K^+ efflux at 0° C, however, is too small for an accurate estimate of the efflux kinetics to be made. But this is possible with the isotope $^{86}Rb^+$ as a tracer for K^+ efflux. The results obtained with yeast preloaded with $^{86}Rb^+$ are shown in Fig. 1. The release of $^{86}Rb^+$ from non-metabolizing yeast cells into the medium increases more than proportionally with time as long as the concentration of dinitrophenol inside the cells increases. No further increase in the rate of Rb^+

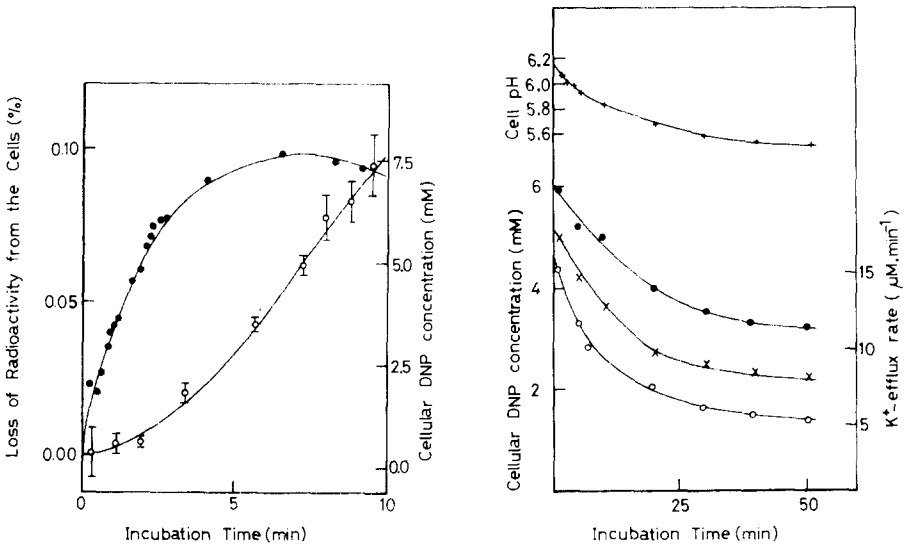

Fig. 1. Comparison of the kinetics of 2,4-dinitrophenol (DNP) induced Rb^+ efflux from non-metabolizing yeast and the time course of dinitrophenol uptake at 0°C. The cells are preloaded with carrier-free ^{86}Rb. ○, Efflux of ^{86}Rb measured as radioactivity released to the medium and expressed in percents of the radioactivity present at zero time in the cells. ●, Total cellular concentration of dinitrophenol. The length of the bars equals the standard error of the mean. The initial dinitrophenol concentration in the medium is 0.1 mM.

Fig. 2. Time course of changes in intracellular dinitrophenol concentration, cell pH and rate of K^+ efflux at 25°C. The concentration of free dinitrophenol is calculated by dividing the concentration of total dinitrophenol in the cell by $(1 + f_{ads})$. The coefficient f_{ads} represents the adsorption coefficient of dinitrophenol [8]. The rate of the K^+ efflux refers to the change in the concentration of K^+ in the medium. +, cell pH; ●, total dinitrophenol concentration, ×, 10 times the free dinitrophenol concentration; ○, K^+ efflux rate in $\mu M \cdot min^{-1}$ or $nmol \cdot ml^{-1} \cdot min^{-1}$.

efflux is found when the intracellular concentration of dinitrophenol does not increase anymore. The ATP content of the cells decreased to 82% ±8 (S.E. with $n = 10$) after 8 min incubation with dinitrophenol. This decrease is too small for an accurate estimate of the kinetics of ATP hydrolysis. The ATP content of the control is 1.1 mM.

At room temperature uptake in yeast is completed already within one minute [11]. After that time the dinitrophenol concentration inside the cells decreases again parallel to a decrease in the cell pH, see Fig. 2. The decrease in the cellular dinitrophenol concentration is accompanied by a decrease in the rate of K^+ efflux. A great part of the dinitrophenol accumulated into the cells is bound to yeast cell constituents. Therefore only a small part of the dinitrophenol inside the cells is free. The extent of binding decreases with the cell pH [8]. As shown in Fig. 2 a correlation exists as well between the concentration of free intracellular dinitrophenolate and the rate of K^+ efflux.

The uptake of salicylic acid in non-metabolizing yeast proceeds far more slowly than the uptake of dinitrophenol, see Fig. 3. The rate of K^+ efflux increases as long as the cellular salicylate concentration increases. The ATP content of the cells decreased to 80% ± 6 (S.E. with $n = 10$) within 80 min.

Fig. 4 shows that the sensitivity of the yeast to dinitrophenolate accumu-

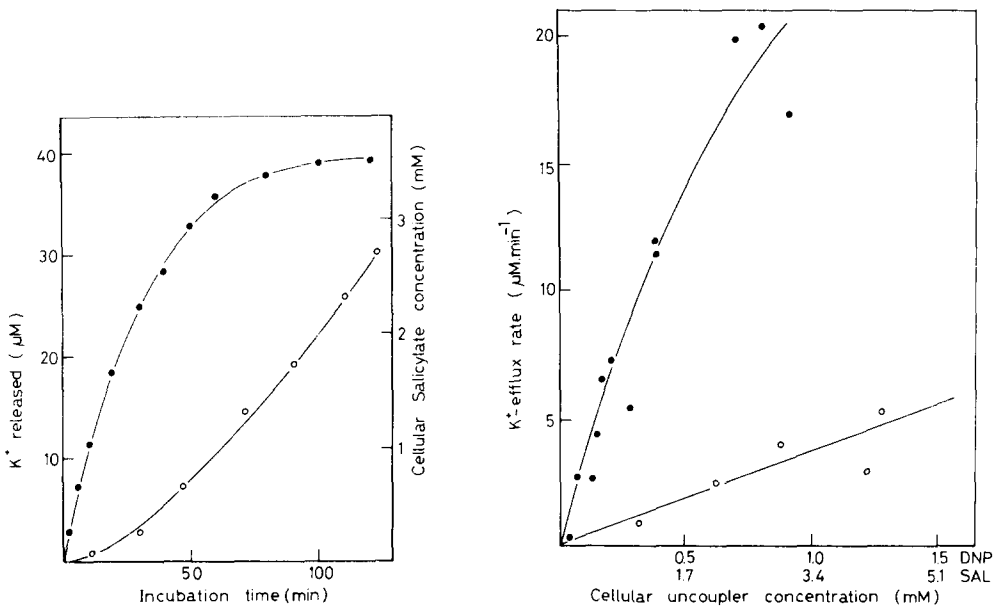

Fig. 3. Time course of salicylic acid uptake and K^+ efflux induced by salicylate at 12°C. The K^+ released to the medium is corrected for changes in K^+ concentration in the absence of salicylic acid. The initial salicylic acid concentration is 0.1 mM. ○, K^+ released to the medium; ●, total cellular salicylate concentration.

Fig. 4. Dependence of the K^+ efflux rate upon the concentration of free dinitrophenolate or salicylate inside the cells at 25°C. The K^+ efflux rate is expressed in the increase in the concentration of K^+ in the medium and is corrected for the efflux of K^+ in the absence of added uncoupler. The K^+ efflux rate is determined after incubation of the yeast with dinitrophenol both during 5 and 60 min and after incubation of the yeast with salicylic acid for 10 min. The cellular concentrations of dinitrophenolate and salicylate are corrected for adsorption of the uncouplers to cellular constituents, see also subscript to Fig. 2. f_{ads} for salicylic acid amounts to approx. 3.5. The concentrations of dinitrophenol added to the medium ranged from 0.01 mM to 0.3 mM (●), and those of salicylic acid from 0.1 mM to 0.5 mM (○).

lated in the cells is much greater than the sensitivity to salicylate. The slope of the tangent to the curve near zero uncoupler concentration is 70 times larger for dinitrophenol than for salicylate.

We have also examined whether the differences in effect caused by salicylate and those caused by dinitrophenol may be traced to differences in the extent of depolarization of the cell membrane. The equilibrium partition ratio of the lipophilic cation dibenzyldimethylammonium has been determined as a function of the dinitrophenol concentration or salicylate concentration. Fig. 5 shows that a single relation exists between the rate of K^+ efflux and the partition of dibenzyldimethylammonium, irrespectively whether the dibenzyldimethylammonium partition is affected by dinitrophenol or by salicylate.

We have finally examined whether the equilibrium distribution ratio of salicylic acid between metabolizing yeast cells and medium is lower than the distribution ratio between non-metabolizing cells and medium as has been found for 2,4-dinitrophenol [8]. This appeared to be true, the values are 5 and

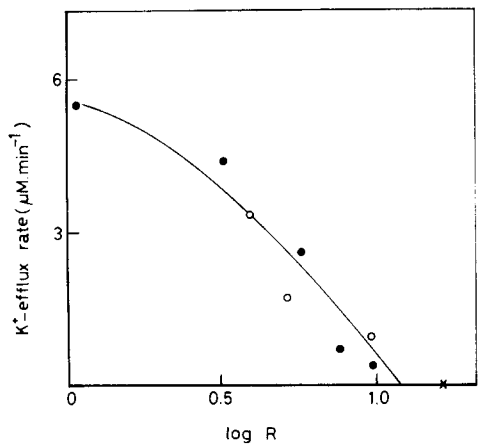

Fig. 5. The relation of the K^+ efflux rate and the equilibrium partition ratio of dibenzyldimethylammonium between the cells and medium. The K^+ efflux rates are corrected for the rate of K^+ efflux found in the absence of added uncoupler. The K^+ efflux rates are determined after incubating the cells for 60 min with uncoupler. R is the partition ratio of dibenzyldimethylammonium. •, dinitrophenol; ○, salicylic acid; X, control value. The dinitrophenol concentrations applied are 0.03, 0.1 and 0.30 mM and those of salicylic acid are 0.1, 0.3, 0.5 and 0.8 mM.

30 respectively, for 10^{-6} M salicylic acid and 40 and 200 for 10^{-6} M dinitrophenol, whereas the distribution ratios of 10^{-6} M dibenzyldimethylammonium amount to 230 and 16, respectively (see also Ref. 6).

Discussion

The use of ^{86}Rb and a tracer for K^+ efflux is probably justified. In contrast with *E. coli* [12], there are no indications that Rb^+ is translocated in yeast in a way essentially different from K^+. The kinetics of Rb^+ efflux from metabolizing yeast observed in the presence of dinitrophenol is qualitatively similar to the kinetics of K^+ release induced by dinitrophenol (unpublished experiments).

The correlation found between the cellular uncoupler concentration and the rate of K^+ efflux, both with the rapidly penetrating dinitrophenol and with the slowly penetrating salicylic acid supports the view that the uncouplers stimulate K^+ efflux from non-metabolizing yeast only after being accumulated into the cells. Apparently the site of action of the uncouplers is inside the cells. Our findings can be interpreted by a model according to which the uncoupler after being accumulated into the cells diffuses back as anion to the medium. This leads to depolarization of the cell membrane and consequently to an increase in the rate of K^+ efflux. In accordance with this view the increased efflux of cellular K^+ is accompanied by a decrease in the partition ratio of dibenzyldimethylammonium, a compound which is thought to be distributed passively across biological membranes according to the Nernst equation [13—17].

It cannot be excluded whether changes in permeability to K^+ play a role in the stimulation of K^+ efflux [18]. Attempts to examine the effect of dinitrophenol upon the rate of $^{86}Rb^+$ influx into non-metabolizing cells only showed that the influx rate is too low to determine this rate with sufficient accuracy.

At least no detectable increase in Rb^+ influx occurs on adding 0.1 mM dinitrophenol to the yeast cell suspension at 25°C. In addition one should realize that an increase in the K^+ permeability cannot be the only cause for the stimulation of K^+ efflux, because this would lead to a more negative membrane potential instead of to a more positive membrane potential as indicated by the partition of dibenzyldimethylammonium.

The hypothesis that the uncouplers pass the yeast cell membrane in the anionic form is in accordance with current views on the mechanism by which uncouplers increase the conductivity of artificial membranes. It is generally assumed that either the anionic form or a complex of anion and acid is able to pass the membrane. Whereas the latter probably applies to artificial membranes [19—21], the former may apply to cellular membranes [22].

According to the model developed one would expect that if the membrane potential becomes more negative (from inside to outside) both dinitrophenolate and salicylate will permeate more rapidly from the cells to the medium, which will give rise to a decrease in the 'equilibrium' distribution ratio of the uncouplers. Accordingly, the distribution ratios of the two uncouplers are lower in metabolizing cells than in non-metabolizing cells, whereas the distribution ratio of dibenzyldimethylammonium is just higher in metabolizing cells than in non-metabolizing cells.

The low decreases in cellular ATP content observed on adding dinitrophenol or salicylic acid refer to the total ATP content. Since the plasma membrane ATPase is not stimulated by dinitrophenol and only the mitochondrial ATPase (personal communication of W. Derks), the decrease in cytoplasmatic ATP may be even less. Therefore it is rather improbable that a decrease in cell ATP contributes much to the apparent depolarisation caused by the uncouplers.

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