Hyperpolarization and intracellular acidification in *Trichoderma viride* as a response to illumination

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Using indirect methods based on uptake of [^3H]tetraphenylphosphonium cation and [^{14}C]benzoic acid by cells of the fungus *Trichoderma viride* we found that the illumination-induced transient hyperpolarization of the plasma membrane is followed immediately by a rapid temporary decrease in intracellular pH. Hyperpolarization and intracellular acidification were completely suppressed by 150 mM-KCl and by the K^+ -ionophore valinomycin. The light-induced acidification of the cytoplasm was not observed in the presence of the cytochrome respiratory chain inhibitors antimycin A and mucidin. Based on these results, we hypothesize that the hyperpolarization of the cells is the consequence of an efflux of K^+ through a light-activated K^+ -channel in the plasma membrane. The loss of positive charge in the cytoplasm caused by this efflux of cations is counterbalanced by H^+ originating from the light-activated mitochondrial respiratory chain.

Introduction

It has become increasingly evident that movements of various ions across cellular membranes constitute an important component of the intracellular signalling pathways involved in regulation of diverse life processes, including the response of cells to various external stimuli (Krueger, 1989; Schroeder & Hedrich, 1989).

In many fungi, e.g. *Trichoderma viride*, the illumination of mycelia with visible or near-UV light regulates differentiation by inducing the formation of conidia (Gressel & Rau, 1983; Dahlberg & Van Etten, 1982). Among the rapid biochemical changes occurring in mycelia as a response to illumination are the transient increase in membrane potential ($\Delta\psi$) and the rise of intracellular levels of ATP and cyclic AMP (Grešík *et al.*, 1988). The increase of the ATP concentration in mycelia could be accounted for by a rapid burst of respiratory activity induced by illumination (Sulová *et al.*, 1990).

In the present study, using indirect methods, we investigated the relationship between the light-induced hyperpolarization of the plasma membrane and the accompanying intracellular acidification.

Methods

Organism and growth conditions. *Trichoderma viride* Person ex S. F. Gray, strain no. F-534 from the Czechoslovak Collection of Microorganisms (T. G. Masaryk University, Brno, Czechoslovakia), was maintained on basal medium agar slants, pH 4.9, containing (per litre): yeast extract, 2 g; KH_2PO_4 , 2 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g; glucose, 20 g; agar, 20 g. Conidia from 7-d-old slant cultures were suspended in basal medium without agar and filtered through a sintered glass filter (pore size 100–200 μm) to remove the mycelium. The concentration of conidia was adjusted to $5 \times 10^8 \text{ ml}^{-1}$ and glass microfibre filter discs (Whatman GF/A, 26 mm diam.) were inoculated centrally with 5 μl of conidial suspension per disc and placed onto the surface of basal agar medium. Petri dishes containing the inoculated discs were wrapped in aluminium foil and incubated at 30 °C for 36 h.

Mycelia were photoinduced as previously described (Farkaš *et al.*, 1985). All operations requiring darkness were done under a red safelight. The photoresponsiveness of mycelia largely depended on their age and physiological state. It is important to note that, in order to obtain a good photoresponse, the mycelium should be in immediate contact with the air (Farkaš *et al.*, 1985). There should be no residual moisture on the surface of agar and, by visual inspection, the mycelium should be velvety and dry, with the margin of the colony reaching just to the rim of the filter disc. Mycelia younger than 32–36 h could not usually be photoinduced. On the other hand, mycelia that were overgrown sometimes conidiated spontaneously, starting from the centre of the colony.

Membrane potential and intracellular pH. The changes in plasma membrane potential ($\Delta\psi$) and intracellular pH were determined by indirect methods by following the rate of uptake of [^3H]tetraphenylphosphonium ion into the cells (Grešík *et al.*, 1988) and by measuring the accumulation of [^{14}C]benzoic acid in the cells, respectively (Valle *et al.* 1986, 1987).

Abbreviations: $\Delta\psi$, plasma membrane potential; TPP^+ , tetraphenylphosphonium ion.

Reproducibility of results. The graphs presented show representative results chosen from at least five independent experiments. Each point is the mean value of three parallel samples; the significance of changes was evaluated by means of Student's *t*-test.

Chemicals. [^3H]Tetraphenylphosphonium bromide ([^3H]TPP $^+$), specific radioactivity 24 Ci mmol $^{-1}$ (888 GBq mmol $^{-1}$), and [^{14}C]benzoic acid, specific radioactivity 20 mCi mmol $^{-1}$ (740 MBq mmol $^{-1}$) were purchased from Amersham. Valinomycin and antimycin A were from Sigma. Mucidin was kindly provided by Dr J. Šubík, Food Research Institute, Bratislava, Czechoslovakia. All other chemicals used were of analytical grade.

Results and Discussion

Measurement of $\Delta\psi$ and intracellular pH

Our previous observations (Farkaš *et al.*, 1985; Grešík *et al.*, 1988, 1989) have indicated that the reception of the light signal by dark-growing cells of *T. viride* is accompanied by a complex set of physiological and metabolic changes, comprising a transient increase in $\Delta\psi$ and an increase in intracellular levels of ATP and cAMP.

In the present work, we tried to explain the mechanism by which the light-induced hyperpolarization occurs. For technical reasons, it was not possible to make direct measurements of $\Delta\psi$ and/or the intracellular pH by means of specific micro-electrodes. Such techniques would require the illumination of hyphae during the introduction of micro-electrodes, i.e. prior to measurement itself.

As an indirect method of assessing the changes in $\Delta\psi$, we measured the rate of uptake into the cells of the lipophilic cation [^3H]TPP $^+$. A critical evaluation of this method was given by Eraso *et al.* (1984). Accordingly, the rate of [^3H]TPP $^+$ uptake into the cells is proportional to $\Delta\psi$, and the constant rate of [^3H]TPP $^+$ uptake reflects the

stability of $\Delta\psi$. In yeast cells, and possibly also in other eukaryotic cells, uptake of TPP $^+$ takes place in two phases: the rate of TPP $^+$ uptake during the first phase is related to $\Delta\psi$ and the second phase reflects the incorporation of TPP $^+$ into intracytoplasmic compartments. In our experiments, the duration of the first phase was about 45 min.

To measure changes in intracellular pH we used a method based on accumulation by the cells of the weak acid [^{14}C]benzoic acid (De la Peña *et al.*, 1982). With this method, the decrease in intracellular pH when the distribution of acid between the cells and medium had reached equilibrium would cause a reduction in the degree of dissociation of labelled acid in the cytoplasm and consequent diffusion of re-associated molecules of [^{14}C]benzoic acid out of the cells (Valle *et al.*, 1986). In our experiments, it took about 12 min to reach equilibrium when the cells were saturated with [^{14}C]benzoic acid.

Owing to the fact that the cells had to be cultivated and illuminated on the surface of solid medium, it was not possible to determine the distribution of [^3H]TPP $^+$ and [^{14}C]benzoic acid between medium and the cells and to calculate the absolute values of internal pH and $\Delta\psi$. For this reason, the measured values should be taken only as a qualitative illustration of the changes in $\Delta\psi$ and intracellular pH evoked by illumination.

Role of K^+

The rapid hyperpolarization of the plasma membrane immediately after the onset of illumination, observed as an abrupt increase in the rate of [^3H]TPP $^+$ incorporation into the cells (Fig. 1), could be explained as the result of

Fig. 1

Fig. 1. Effect of KCl on time-dependent changes of $\Delta\psi$ expressed as uptake of [^3H]TPP $^+$ into cells of *T. viride*. ●, Illuminated control mycelium; ●, illuminated mycelium in the presence of 150 mM-KCl; ○, dark control. Illumination started at the time indicated by the arrow. All operations requiring darkness were done under a red safelight.

Fig. 2

Fig. 2. Time-dependent changes in the rate of [^3H]TPP $^+$ accumulation in *T. viride* in the presence of 50 μM -valinomycin. ●, Illuminated mycelium; ○, dark control. Other conditions were as in Fig. 1.

Fig. 3

Fig. 4

Fig. 5

Fig. 3. Time-course of [^{14}C]benzoic acid accumulation by *T. viride*. ●, Illuminated mycelium; ○, dark control. Illumination started at the time indicated by the arrow.

Fig. 4. Effect of antimycin A (○) and mucidin (●) (both at $10\ \mu\text{g ml}^{-1}$) on accumulation of [^{14}C]benzoic acid by *T. viride*. Mycelia were pre-incubated with [^{14}C]benzoic acid and the effectors for 12 min before illumination. Illumination started at the time indicated by the arrow.

Fig. 5. Effect of KCl (●) and NaCl (○) (both at 150 mM) on accumulation of [^{14}C]benzoic acid by *T. viride*. Mycelia were illuminated at the time indicated by the arrow.

an efflux of positively charged ions from the cytoplasm into the cell exterior or by a movement of negative charge from the extracellular space into the cytoplasm. The likely candidates are H^+ , K^+ , Ca^{2+} and/or Cl^- . Since our preliminary results have shown that the K^+ -ionophore valinomycin was able to abolish the effect of light on $\Delta\psi$, we investigated the role of K^+ more closely.

The intracellular concentration of K^+ in fungal cells lies in the range 100–300 mM (Serrano, 1985). Fig. 1 shows that the presence of 150 mM-KCl in the medium during photoinduction not only completely prevented hyperpolarization of the plasma membrane but caused a temporary decrease in $\Delta\psi$ after illumination. Lower concentrations of KCl, or an equimolar concentration of NaCl in the external medium were without effect on the hyperpolarization, and the time-course of [^3H]TPP $^+$ uptake during illumination in the presence of NaCl was the same as in the illuminated control.

This behaviour can be interpreted as follows. Illumination triggers the opening of a specific K^+ -channel in the plasma membrane and K^+ leaks out from the cells along the concentration gradient. The redistribution of K^+ would result in an increase in $\Delta\psi$, positive outside. However, with a sufficiently high concentration of KCl in the external medium, the gradient of K^+ would be levelled-off or reversed and the efflux of K^+ from the cells would be prevented (Van de Mortel *et al.*, 1988).

An effect similar to that of external K^+ was observed with 50 μM -valinomycin present in the medium at the time of photoinduction. In the dark, valinomycin

markedly lowered $\Delta\psi$, while after illumination there was a temporary decrease in $\Delta\psi$ as indicated by the loss of accumulated [^3H]TPP $^+$ from the cells (Fig. 2).

Role of H^+

As can be seen in Fig. 1, the disturbance of $\Delta\psi$ caused by illumination was restored after about 2 min. This may be explained by a reversal of the K^+ flux or by replacement of extruded cytoplasmic K^+ by some other cation(s). It is very probable that K^+ in the cytoplasm was replaced by H^+ . This assumption stems from the observation that approximately 2 min from the onset of illumination, an internal acidification took place, manifested as the loss of accumulated [^{14}C]benzoic acid from the cells (Fig. 3).

The acidification which follows the hyperpolarization could be the result of K^+/H^+ symport from the external medium, as has been described for *Neurospora crassa* (Takeuchi *et al.*, 1988). Another possibility is that the H^+ that caused the internal acidification were not imported from the outside, but were generated inside the cytoplasm or released into the cytoplasm from some internal compartment. In a previous report (Sulová *et al.*, 1990) we reported that illumination of dark-grown mycelia of *T. viride* caused an oxidative burst accompanied by transfer of electrons along the cytochrome respiratory chain. Since the stimulated oxygen consumption during the oxidative burst was not accompanied by increased production of CO_2 the possibility that internal acidification was due to dissociation of H_2CO_3 or of organic acids seems improbable.

The possibility exists that H^+ were released into the cytoplasm by respiring mitochondria. According to the chemiosmotic theory (Mitchell, 1961), the transfer of electrons along the respiratory electron-transport chain is accompanied by translocation of H^+ from the mitochondrial matrix across the inner membrane into the mitochondrial intermembrane space. Since the outer mitochondrial membrane is permeable to ions and various low-molecular-mass solutes, H^+ that have been massively discharged from the mitochondrial matrix during the light-induced oxidative burst could reach the cytoplasm and cause a transient acidification. The massive discharge of H^+ from mitochondria would result in their hyperpolarization which, according to chemiosmotic theory, could be used as a driving force to generate ATP. A doubling in the intracellular level of ATP following illumination has been reported previously (Farkaš *et al.*, 1985; Grešík *et al.*, 1988).

In order to prove the involvement of respiration in the intracellular acidification, we examined the effect of antimycin A and mucidin, two inhibitors of respiration that act on the electron-transport chain. Both compounds inhibit the transfer of electrons at the level of the cytochrome *b/c*₁ complex, although the mechanism of their action is different (Šubík *et al.*, 1974). Fig. 4 shows that both antimycin A ($10 \mu\text{g ml}^{-1}$) and mucidin ($10 \mu\text{g ml}^{-1}$) prevented the illumination-induced internal acidification.

As in the case of $\Delta\psi$, 150 mM-KCl present in the medium at the time of illumination suppressed the light-induced pH change (Fig. 5). On the other hand, the addition of an equimolar concentration of NaCl had no effect on the internal acidification.

Effect of external K^+ on conidiation

To confirm the physiological role of K^+ in the process of light-induced conidiation, we compared the effect of external K^+ , Na^+ and/or Ca^{2+} on conidiation. Mycelia that had been grown in the dark on the surface of basal medium for 36 h were transferred onto media supplemented with physiological concentrations of KCl (150 mM), NaCl (150 mM) and/or $CaCl_2$ (25 mM) (Serrano, 1985). Controls were placed onto the basal medium without additions. After 15 min pre-incubation in the dark, the mycelia were illuminated (white fluorescent light, 2 klx) for 1 min and transferred back to the agar plates with basal medium and allowed to conidiate for 48 h in the dark. Photoinduction did not occur in mycelia illuminated in the presence of 150 mM-KCl whereas conidiation proceeded normally in controls and in mycelia illuminated in the presence of 150 mM-NaCl and/or 25 mM- $CaCl_2$.

Working hypothesis

On the basis of these observations, and in accordance with our previous findings, the following working hypothesis was constructed. Illumination opens, by a so-far unknown mechanism, specific K^+ -channels in the plasma membrane. As a consequence, the efflux of K^+ along the concentration gradient causes a hyperpolarization of the plasma membrane. The disturbed charge homeostasis in the cells resulting from the efflux of K^+ is compensated by H^+ discharged into the cytoplasm from light-activated mitochondria, thereby causing the acidification of cytoplasm. The electrochemical gradient for H^+ created between the inner membrane of mitochondria and the cytoplasm is used to drive the production of ATP whose intracellular concentration rapidly increases after the onset of illumination (Farkaš *et al.*, 1985; Grešík *et al.*, 1988).

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