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Effect of induced changes in membrane permeability on the defence response of *Chlorella vulgaris* to infection by *Acholeplasma laidlawii*☆

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Abstract

The defence response of photosynthesising cells to the stress of infection by mycoplasma was investigated using an algae model of *Chlorella vulgaris* exposed to the Mollicute, *Acholeplasma laidlawii*. The first line of the defence is at the interface between the pathogen and its host is at the cell wall and its subjacent plasma membrane. Thus, one of the important non-specific changes in cells on pathogen stress is the change of membrane permeability that in turn disturbs ion homeostasis. For a more complete understanding of the mechanisms of the cell response to the infection, three known effectors of ion permeability in the plant cell plasma membrane were used, namely Gd^{3+}, RNase, and ATP. Gd^{3+} is a Ca^{2+}-channel inhibitor and possibly affects other ion channels in the plasma membrane. It is thought that the action of RNase may be through low molecular fragments formed by the hydrolysis of high molecular RNA degradation. ATP in the living cell not only has an energetic role but also takes part directly, for instance through cyclic AMP, and/or indirectly in intracellular metabolic signalling, resulting in the activation of the reactions involved in phosphorylation, catalyzed by the membrane kinases. It is considered that changes in the distribution of ions between the cytoplasm and the external environment alter the energy metabolism of the cells because ion homeostasis is an active energy-requiring process, which is tightly related to the intensity of its defence reactions.

The results showed that the changes in the energy metabolism of the cells depend on the requirements of them for coping with changes of the environment. Thus, the separate application of Gd^{3+}, RNase and ATP had an insignificant effect on the rates of heat production and oxygen consumption in the dark, photosynthesis measured as oxygen evolution, generation of superoxide on the *Chlorella* cells, grown under the optimal condition. On the other hand, the rate of the energetic processes significantly increased when infected *Chlorella* were challenged separately by the three compounds. It was concluded that an additional expenditure of metabolic energy necessary for the defence of microalgal cells in stress conditions.
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Keywords: Cell signalling; Ion homeostasis; Superoxide anion; Dark respiration; Photosynthetic rate

1. Introduction

Plant defence is currently an important field for study, not least because of its implications for agriculture [1]. *Chlorella* is extensively used as a suitable model for investigating this problem [2–5] because of its structural and physiological similarity of these protists with higher plants. To mimic the defensive response to infection, we have been using a model system in which the microalgal cells of *Chlorella vulgaris* are challenged with the cells of the mollicute (mycoplasma), *Acholeplasma laidlawii* [6,7]. According to these studies, the *Chlorella* cells reacted to the presence of the mycoplasma by the enhanced production of active oxygen species, which was manifest by simultaneous increases in the rates of heat production to give the so-called heat burst, oxygen uptake in the dark to give the respiratory burst and oxygen evolution as an indicator of the apparent photosynthetic rate in the light. It

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is important to note that the cascade of stress responses of infected *Chlorella* cells with the mycoplasma was similar to that found in higher plants [8,9].

It is obvious that the interface between the pathogen and its host is the cell wall and its subjacent plasma membrane. It is known that the initial signals of the awareness of pathogen infection in plants occur at the interaction between secreted elicitors and trans membrane receptors of both the cell wall and the plasma membrane [10–12]. The latter plays a crucial role in the reception and transduction of environmental signals, which activates an amplified intracellular signal cascade within the cell leading to a particular response. Among the important non-specific changes of plant cells to pathogen stress are:

1. intensification of biopolymers and lipid catabolism;
2. the change of membrane permeability and as a result the disruption of ion homeostasis;
3. the increase of free radicals content;
4. the alterations of energetic processes rate [10–13].

For a more complete understanding of the mechanisms of the cellular response to mycoplasma infection, Gd^{3+}, RNase, and ATP were used to modify the ion permeability of plant cells plasma membrane in the following ways. Gd^{3+} is a specific inhibitor of the mechanosensitive channels in the plasma membrane that does not penetrate into cells [14–16]. It is thought that the action of RNase action may be due to the degradation of high molecular weight RNA to give lower molecular weight RNA that possesses the enzyme activity to affect embrane permeability [17–19]. Thus, RNases may take part in defence reactions to pathogen attack [20,21]. ATP in the living cell carries out not only the energetic role but is important in the formation of such signalling molecules such as cAMP and cGMP as well as phosphorylation cascades, catalyzed by membrane kinases [22–24].

Alterations in the distribution of ions between the cytoplasm and the external environment may cause changes in the energy metabolism of the cells because ion homeostasis is an active, energy-requiring process. In addition, these alterations may cause the initiation of energy-consuming defence mechanisms, which are tightly related to the intensity of the defence reactions [25–27]. The metabolic rate of cells and organisms is directly proportional to the instantaneous heat flow rate [28] and thus applying the calorimetric method ensures a complete description of the catabolic changes accompanying the defence against infection.

If oxygen uptake measurements are undertaken simultaneously with direct calorimetry, then it is possible to discriminate between aerobic and anaerobic metabolic processes. Consequently, measurements were taken of the effects of the agents on the oxygen uptake rate. One of the basic mechanisms of non-specific defence of plants on pathogenic attack is the increase in the production of active oxygen species that include the superoxide anion radical [29–32]. The enhanced generation of the superoxide is likely to have been the most important of the primary defensive responses of the host to the mycoplasma infection. As the viability of the host on infection by the pathogen may be limited by the energetic cost, we studied the rate of photosynthesis. Uniquely, this transforms absorbed light energy into the chemical energy of metabolites and causes the formation of carbohydrates used by organisms as the energy source for catabolic and anabolic processes, with the former providing the energy for the latter.

The aim of this research is to study key energetic defence processes of *Chlorella* cells infected with mycoplasma by the use of specific membrane permeability modulators.

2. Experimental

The unicellular microalga *Chlorella vulgaris* and the Mollicute *Acholeplasma laidlawii* were the objects for this investigation. *Chlorella* cells were grown in Tamiya medium, pH 6.8–7.2 [33] at 30 °C, and illuminated at 1×10^4 lx with a photoperiod of light/dark of 12/12 h. Cell suspensions were bubbled with 0.3% CO_2 in air. The optical density was maintained at (1–1.5×10^8 cells/ml).

A. laidlawii was grown at 37 °C in test tubes using the Edward medium [34]. Before each experiment, the mycoplasma suspension was centrifuged at 20,000× *g* in phosphate buffer for 10 min. Mycoplasma were re-suspended in the buffer with a titre of 10^7 ml^{-1} viable cells and added to the cultures of chlorella in Tamiya medium at the ratio of 1:4. The numbers of living mycoplasma were determined both spectrophotometrically by measuring the culture density and observationally by isolating the mycoplasma on agar-medium, counting the colonies and expressing the culture titre as colony forming units (CFUs).

When appropriate, $Gd(NO_3)_3$ (5×10^{-4} M), RNAse (3×10^{-5} M), or ATP (1×10^{-3} M) were added to the *Chlorella* suspension. The microalgae were challenged with the mycoplasma suspension in the ratio 4:1 after incubation of the cells for 20 min with the above agents.

The samples were then divided into three aliquots in order to measure: (i) the rate of heat production; (ii) the rate of oxygen uptake/evolution; and (iii) the amount of superoxide formation with time.

The instantaneous rate of heat production was measured in the heat conduction LKB batch bioactivity monitor (BAM – the direct calorimetric successor is the thermal activity monitor (TAM) manufactured by Thermometric AB, Jarfalla, Sweden [35]). Suspensions of 1.5 cm^3 were placed in unstirred 3 cm^3 glass vessels that were hermetically sealed before thermal equilibration for 15–20 min. This means that the first point for measuring the rate of heat production in the dark corresponded to 40 min (20 min incubation the cells with agents + 20 min for thermal equilibration of vessels) after adding the mycoplasma to the *Chlorella* culture.

The oxygen uptake and evolution rates were measured by the polarographic method using a Clark-type electrode. Each 3.2 ml sample was placed in a measuring tube located in a tight-fitting water bath and equilibrated to 30 °C in 5 min.

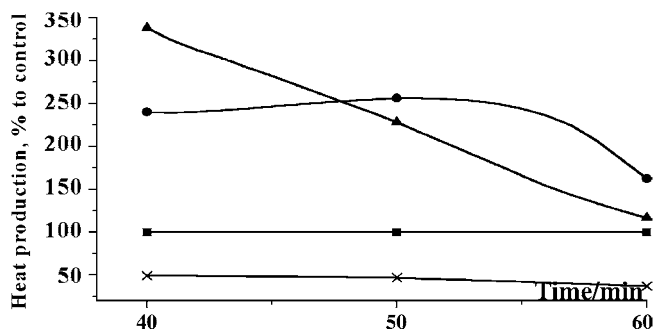

Fig. 1. The action of Gd^{3+} ions on the rate of heat production of *Chlorella* cells infected with mycoplasma. (■) *Chlorella*, (●) *Chlorella* + mycoplasma, (×) *Chlorella* + Gd, (▲) *Chlorella* + Gd + mycoplasma.

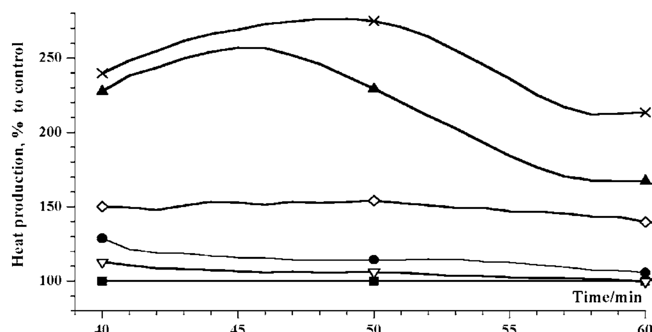

Fig. 2. The instantaneous rate of heat production of *Chlorella* infected with *A. laidlawii* after exposure to RNase and ATP. (■) *Chlorella*, (▲) *Chlorella*+ mycoplasma, (●) *Chlorella* + RNase, (×) *Chlorella* + RNase + mycoplasma, (▽) *Chlorella* + ATP, (◇) *Chlorella* + ATP + mycoplasma.

Then a black box was fitted over the bath to allow measurement of the oxygen uptake rate in the dark for 3 min. The box was removed and the cells were illuminated to record the rate of oxygen evolution for a further 3 min period.

The quantity of superoxide in the infected *Chlorella* cultures was estimated spectrophotometrically at 490 nm wavelength using 1 mM epinephrine as described in [36]. The suspension was incubated with epinephrine for 10 min.

The experiments were repeated 3 times and the standard error was calculated (Microcal™Origin™,V: 5,0) for the data.

3. Results

As can be seen in Fig. 1, as soon as the vessels were equilibrated to 37 °C it could be seen that the instantaneous rate of heat production of the *Chlorella* cells treated with mycoplasma was 250% greater than in the control. The rate of heat production of algal cells treated with Gd^{3+} was 15–20% lower than the control 40 min after addition of Ga^{3+} to the *Chlorella* suspension and this difference continued throughout experiment period. The amount of heat production by *Chlorella* suspension treated by action of pathogen with Gd^{3+} was at first somewhat higher than the control but then sharply decreased to an amount close to the control level. It should be noted that this effect was considerably less than resulted when the host contacted the pathogen.

Data for the effect of RNase and ATP on the rate of heat production of plant cells at mycoplasma infection are presented in Fig. 2 in terms of difference from controls. The "heat burst" by *Chlorella* cells infected with mycoplasma was observed in response to the introduction of RNase. The experiments showed that the rate of the heat production in the mixed culture subjected to RNase was slightly greater by 12% than in the suspension of *Chlorella* with mycoplasma after incubation for 40 min.

The effect of ATP on the rate of heat production of *Chlorella* infected with *A. laidlawii* was very similar to that for RNase except that the increase of the rate of heat production of the *Chlorella* suspension was smaller than on addition of RNase to a similar preparation. It is interesting to note that RNase and ATP on their own had an insignificant effect on the rate of heat production.

The "heat burst" of the *Chlorella* suspension on the action of the above agents was accompanied by an increase of O_2 uptake. As seen in Fig. 3, the rate of oxygen uptake was much higher than that of the control after addition of *A. laidlawii*. The addition of Gd^{3+} to the *Chlorella* suspension inhibited the oxygen uptake rate by 15–20%. Gd^{3+} ions caused a stimulatory effect on the rate of oxygen uptake by the *Chlorella* suspension infected by mycoplasma. It should be noted that the amounts of the oxygen uptake rate by *Chlorella* on addition of mycoplasma and Gd^{3+} ions were less than infected algal cells. But the combined effect of Gd^{3+} and the pathogen on the oxygen uptake was greater than by the action of Gd^{3+} ions on its own.

The addition of RNase and ATP to the infected *Chlorella* suspension resulted in a significant increase in the rates of oxygen uptake, RNase by 80% and ATP about 50% (Fig. 3) compared with the control.

The data depicted in Fig. 4 show that the amount of superoxide anion produced by the *Chlorella* suspension mixed

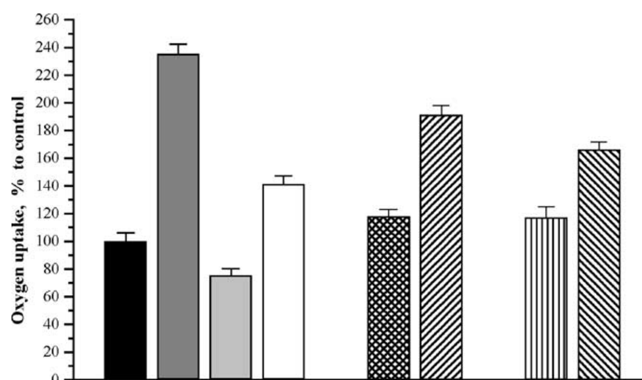

Fig. 3. The effect of Gd^{3+} ions, RNase and ATP on the rate of oxygen uptake by *Chlorella* exposed to mycoplasma. (■) Control, (▒) *Chlorella* + mycoplasma, (□) *Chlorella* + Gd, (□) *Chlorella* + mycoplasma + Gd, (▩) *Chlorella* + RNase, (▨) *Chlorella* + mycoplasma + RNase, (Ⅲ) *Chlorella* + ATP, (▧) *Chlorella* + ATP + mycoplasma.

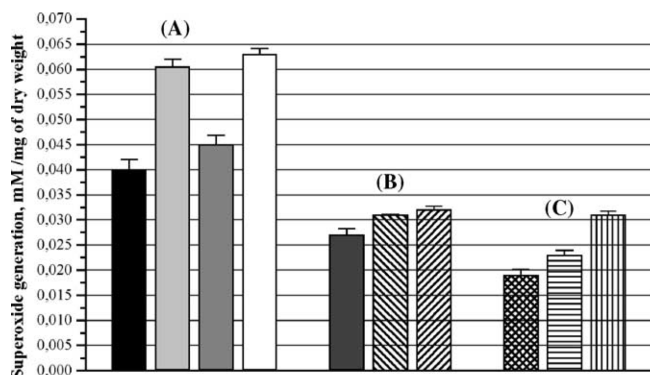

Fig. 4. Intensity of superoxide formation in *Chlorella* cells treated with mycoplasma on exposure to the modulators of membrane conductivity. (■) *Chlorella*, control, (▭) *Chlorella* + mycoplasma, (▭) *Chlorella* + Gd, (□) *Chlorella* + mycoplasma +Gd, (■) *Chlorella*, control, (▧) *Chlorella* + RNase, (▨) *Chlorella* + mycoplasma + RNase, (▩) *Chlorella*, control, (▤) *Chlorella* + ATP, (▥) *Chlorella* + mycoplasma + ATP.

with *A. laidlawii* was almost 50% higher than the control and in the combination of Gd^{3+} ions with the pathogen. Although Gd^{3+} ions increased by 10–15% the amount of superoxide produced by the *Chlorella* culture, it had no effect on the superoxide production by the culture challenged with mycoplasma. A similar result was obtained for the treatment of the cultures with RNase (see Fig. 4). The enzyme had a small effect on superoxide production in the mixed culture. On the other hand, ATP produced a 50% increase in superoxide generation compared with the microalgal control. A significant increase in the rate of superoxide generation by the microalgal cells resulted from the joint action of pathogen and ATP (Fig. 4).

In terms of the net rate of oxygen evolution, the data in Fig. 5 show that Gd^{3+} had little effect. However, oxygen evolution was reduced by 10–25% when Gd^{3+} was present in mixed cultures. On the other hand, the rate of oxygen evolution increased by up to 40% in mycoplasma-infected

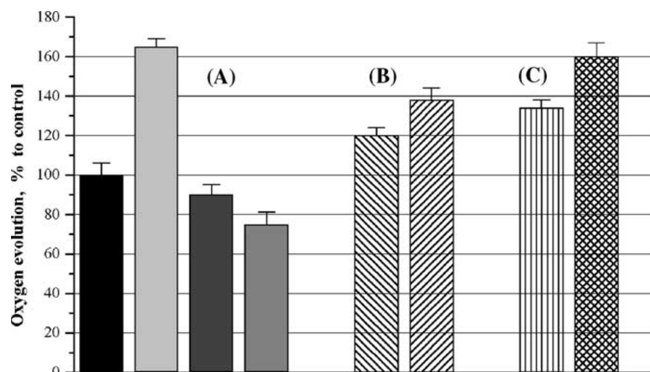

Fig. 5. The oxygen evolution of the *Chlorella* suspension infected by mycoplasma on addition Gd^{3+}, RNase and ATP. (■) *Chlorella*, (□) *Chlorella* + mycoplasma, (■) *Chlorella* + Gd, (▭) *Chlorella* + mycoplasma + Gd, (▧) *Chlorella* + RNase, (▨) *Chlorella* + mycoplasma + RNase, (▥) *Chlorella* + ATP, (▩) *Chlorella* + mycoplasma + ATP.

Chlorella subjected to RNase compared to the control. An even greater increase in oxygen evolution of up to 60% was observed when mixed cultures of *Chlorella* and the pathogen were exposed to ATP (Fig. 5). This may have been due partly to the fact that ATP itself also caused an intensification of oxygen evolution.

4. Discussion

As a general feature, photosynthesising organisms react to the action of various pathogens as well as other extreme factors with a cascade of stress responses. The signals of the awareness of the pathogen infection by them are universal. The interaction between elicitors, exocytosed in medium and transmembrane protein receptors of plasma membranes of the host play the role of the initial reaction. The signals trigger the chain of the processes of induction and regulation of phytoimmunity [10,37–39]. The elicitors specific to proteins receptors induced the activity of the intracellular protein molecules of the cascade. G-protein complexes probably initiate this cascade [40,41].

The key role in the regulation of defence reactions of cells belongs to the reactions of phosphorylation–dephosphorylation cycles, which are catalyzed by different protein kinases under stress conditions including the infection by pathogens [9,42,43]. The signal cascade from elicitors to plasma membrane receptors, G-proteins, protein kinases, and factors of transcription regulation under stress conditions constitutes the response of the organisms. As noted above, one of the non-specific but important responses of cells under pathogen stress is the change of cellular membrane permeability resulting in the disruption of ion homeostasis [10,13]. Thus, one approach to investigate mechanisms of defence reaction of plant cells challenged by mycoplasma is the modification of this homeostasis using modulators of ion conductivity of the membrane [44,45].

The disruption of ion homeostasis induces changes in the energy metabolism of the organism that is tightly related to the intensity of the defence reactions. The maintenance of ion homeostasis requires a considerable expenditure of metabolic energy, because the active transport of ions is carried out by numerous ATPases using the energy of ATP. The ability of Gd^{3+} ions to bind critical cell membrane components resulted in its use as a blocker of membrane ion channels. These ions do not penetrate into cell and accumulate at the cell membrane [14–16].

Treatment of infected *Chlorella* with Gd^{3+} resulted in the increase in the rate of formation of superoxide anion radical (Fig. 4), oxygen uptake (Fig. 3), and heat production (Fig. 1) compared to the control, but the magnitude of the latter was considerably less than when the host was mixed with the pathogen. Interestingly, in healthy cells of *Chlorella*, ions of Gd^{3+} caused an insignificant decrease in the oxygen uptake and heat production while the formation of the superoxide was slightly higher than those in the control.

It is thought that Gd^{3+} ions cause the disruption of Ca^{2+} membrane transport and thus alterenergetic processes. Ca^{2+} ions are universal second messengers in signal systems [46–48]. The increase of Ca^{2+} concentration in cytoplasm is one of the primary reactions of cells to the infection of different pathogens. The accumulation of these ions is connected with the membrane Ca^{2+} channels [49]. Gd^{3+} ions block ATPase channels and inhibit the Ca^{2+} exchange, thus disturbing ion homeostasis. This could result in the reduced energy expense for active ion transport that requires ATP synthesized as a result of respiration. In other words, there is less demand for the synthesis of ATP because there is a lesser requirement for the hydrolysis of the substance in active transport. In stress conditions, e.g. upon the infection of *Chlorella* by mycoplasma, it is supposed that additional energy was required in terms of the production of the superoxide anion and hydrogen peroxide to kill the mycoplasma, resulting in the intensification of both oxygen uptake and heat production. The same reduction in the rate of oxygen evolution by *Chlorella* in the light can be caused by the partial destruction of chlorophyll after mycoplasma infection and Gd^{3+} [8].

The addition of RNase to the infected *Chlorella* suspension caused the increase in the rates of heat production, oxygen uptake, oxygen evolution and formation of superoxide radical (Figs. 2–5). The mechanism of RNase action is complex. It is likely that RNases are involved in the degradation of high molecular RNA. In the recent years some evidence has been accumulated about the enzymatic activity of low molecular RNA and their regulatory role of different metabolic processes. It is significant that low molecular RNA is especially activated by the action of different stressors on the plant [17,21,50]. There are data that RNase induces the ion transport system of plasma membrane (including proton ATPases) and as a result stimulating the heat production and the formation of superoxide anion radical in dark and light energetic processes. According to [17,21,50], local changes of the ion composition in cells can trigger the RNase activity and as result the RNA degradation, which is accompanied by stimulation of energy producing systems.

Interestingly, in the *Chlorella* control, RNase caused an insignificant increase in the heat production, oxygen uptake, light oxygen evolution and the formation of superoxide (Figs. 2–5). It is supposed that *Chlorella* cells, growing in optimal conditions, do not require the additional expenditure of metabolic energy as compared with cells under stress conditions.

It has been proposed that exogenous ATP has a positive influence on the defence reaction of *Chlorell*a cells infected by mycoplasma [22,23]. Addition of ATP to the *Chlorella* infected by mycoplasma induced an enhanced superoxide formation (Fig. 4) and, expectedly, the rates of oxygen uptake (Fig. 3) and heat production (Fig. 2). The ATP molecule has a unique importance for living cells, being the main directly used donor of free energy in biological systems and thus allowing cells to survive in extreme environmental conditions. Moreover, it can play a regulatory role and contribute to the cell signaling via phosphorylation of membrane proteins catalyzed by the kinases [22,23].

It seems that the regulatory ATP effect is in the rapid effect on the mechanism of the membrane proteinphosphorylation by protein kinases [22,44,51]. According to [22,23,52], ATP regulates the plasma membrane ion transport. The alteration of the phosphorylation rate in the regulation of plasma membrane ion transport by ATP probably increases the rate of energetic processes. According to [22], energy is needed for the rapid response of the cell to external signals and to sustain the sensitivity to these signals for a prolonged time.

5. Conclusions

Summarizing the data, it can be concluded that the defense reactions of microalgal cells to infection require additional energy. It is proposed that the conditions decreasing the energetic status of plant cells reduce the phytoimmunity of the organisms to the infection. So, Gd^{3+} ions rapidly decreased the heat production rate of the infected *Chlorella*. RNase and ATP, on the other hand, increase respiration and photosynthesis, as seen by the increase in the rate of heat production, and thus probably can affect the stability of cells to the infection.

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