Orthovanadate Induces Phytoalexin Production in Pea Suspension-Cultured Cells

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We previously reported that the addition of orthovanadate suppressed the defense responses of plant differentiated tissues induced by a fungal elicitor. In this report, the effect of orthovanadate on the defense response of pea cultured cells was examined. The activities of ATPase and PI metabolism in plasma membrane fraction, which was prepared from suspension-cultured cells, were inhibited in vitro by orthovanadate as well as those in plasma membranes from pea epicotyl tissue. However, orthovanadate alone induced the accumulation of a phytoalexin, pisatin in suspension-cultured cells of pea in a manner similar to 

CuCl₂. The viability of pea suspension-cultured cells was decreased by orthovanadate as well as 

CuCl₂. These results indicated that orthovanadate acts as an abiotic elicitor to pea 
suspension-cultured cells as observed in those of red bean, peanut and Petunia 
hybrida.

Key words: defense response, elicitor, Pisum sativum, suspension-cultured cells, orthovanadate

Introduction

Plants have an ability to protect themselves against environmental stress such as invasion by phytopathogens. When attacked by pathogens, the plants are able to rapidly recognize 

threats and to establish the various defense responses. These defense responses include accumulation 

of phytoalexins, activation of pathogenesis-related (PR) proteins and in 

solubilization of cell wall-bound proline-rich glycoproteins (for review, see Dixon and Lamb). It was previously reported that a pea pathogen, 

Mycosphaerella pinodes, secreted a high-
molecular-weight elicitor and a low-
molecular-weight glycopeptide suppressor in its 
pycnospora germination fluid. The elicitor from M. pinodes induces pea defense 

responses such as the production of a major 

phytoalexin of pea, pisatin, the activation of PR proteins such as β-1, 3 glucanase and chitinases and the formation of an as yet-unknown infection-inhibitor. However, the concomitant presence of the suppressor from the fungus blocked or delayed these defense responses (for review, see Shiraiishi et al.). One of the major actions of the suppressor was thought to inhibit the ATPase activity. On the other hand, orthovanadate, an inhibitor of P-type ATPases, also blocked the active defense responses of pea 
tissues in a similar way to the suppressor. Thus, inhibition of ATPase activity was thought to be important for the pathogens to avoid the active defense responses in the tissues of the host.

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plant. However, it was reported that vanadate induced defense responses such as phytoalexin accumulation and the activation of key enzymes in the biosynthetic pathway for phytoalexins in suspension-cultured cells of peanut, red bean and Petunia hybrida. In this report, therefore, we examined the effect of orthovanadate on the active defense response of suspension-cultured cells of pea.

Materials and Methods

Chemicals — Fluorescein diacetate was purchased from Sigma Chemicals Co., Ltd., St. Louis, MO. (γ,γ-32P) ATP (6000 μCi/ml) was obtained from Amersham (U.S.A.). Other chemicals were purchased from Wako Pure Chemical Ind., Osaka, Japan.

Cell culture — The suspension-cultured cells of pea (Pisum sativum L., cv. Mldoriumu) used in this study had been maintained by subculturing at 7 day intervals in B5 medium containing 1.5 mg/l of 2,4-D and 0.2% casein enzymatic hydrolysate as described previously. The culture was grown at 22 °C in 100 ml Erlenmeyer flasks containing 30 ml of the medium on a rotary shaker (100 rpm).

Preparation of elicitor from Mycosphaerella pinodes — The elicitor was prepared from the germination fluid of pycnospora of Mycosphaerella pinodes Westergren, strain OMP-1 (IFO-38042, ATCC-43741) as described previously. The concentration of the elicitor was determined by the method of Dubois et al. with glucose as the standard.

Determination of pisatin accumulated in pea suspension-cultured cells — The cell culture (2.1 ml) was aliquoted into 10 ml Erlenmeyer flasks and was treated with 700 μl of test solution, which contained several concentrations of orthovanadate and CuCl2 dissolved in B5 medium. After incubation at 22 °C for 24 h, the amount of pisatin was determined by the method of Masuda et al.

Preparation of the plasma membrane fraction — The plasma membrane fraction was prepared from 1-day-old cultured cells or elicited seedlings by Yoshida et al. The protein content of the respective fraction was determined by the method of Lowry et al. with Bradford with BSA as the standard. The membrane fraction was stored at -80 °C until use.

Determination of ATPase activity in vitro — The activity of ATPase was determined by the method of Perlín and Spanwick. Assay of ATPase was carried out at 37 °C for 60 min in 80 mM Tris-MES buffer (pH 6.3) that contained 1 mM Mg-ATP in the absence or presence of several concentrations of Na3VO4.

Phosphorylation of lipid in vitro — Determination of lipid phosphorylation in vitro was carried out according to the method as described previously. In brief, the reaction mixture was adjusted to 50 μl containing 20 mM Tris-MES (pH 6.3), 15 mM MgSO4, 100 mM GTP, 20 nM [γ-32P] ATP, and 12.5 μg of plasma membrane fraction in the presence or absence of the elicitor (100 μg/ml, glucose equiv.) and Na3VO4 at the concentration of 1 mM or 0.1 mM. The reaction was initiated by the addition of the plasma membrane fraction. After incubation at 6 °C for 20 min, the reaction was terminated by the addition of 200 μl of chilled chloroform/methanol (v/v = 1/2).

Extraction and analysis of phosphorylated lipids — Phospholipids were extracted by the method of Bligh and Dyer with slight modifications. The extract with chloroform/methanol (v/v = 1/2) was centrifuged at 7,000 x g for 15 min to remove the denatured proteins. The supernatant was transferred to an Eppendorf tube and 60 μl of chloroform and 1% (w/v) potassium chloride solution were added. After vigorous mixing, the lower phase was collected and washed with methanol/water (v/v = 10/9). The resultant lower phase containing lipids was re-collect- ed and dried in vacuo. The residue was then
dissolved in 1 μl of chloroform/methanol (v/v = 2:1) and applied onto a silica gel TLC plate (Whatman, K5) that had been presoaked in 1% (w/v) potassium oxalate for 75 s and then dried at 110 °C for 6 h prior to use. The development was carried out using chloroform/methanol/32% ammonia/water (86:76:4:18, by vol.). After development, the plate was exposed to 0.65% (w/v) primuline solution in 80% (v/v) acetone to visualize the lipid compounds. The incorporation of radioactivity from [γ-32P]ATP into phosphatidylinositol monophosphate (PIP) and phosphatidylinositol 4, 5-bisphosphate (PIP2) was determined with a Bio-imaging scanner system (Bas 2000 system, Fuji, Tokyo, Japan).

Measuring of viability of cultured cells by fluorescein diacetate-staining. Viability of cell culture was determined by fluorescein diacetate (FDA)-staining. Three hundred μl of cultured cells were aliquoted into 10 ml Erlenmeyer flasks and incubated at 22 °C for 24 h in B5 medium in the absence or presence of several concentrations of Na2VO3 and CuCl2. After incubation, 300 μl of fluorescein diacetate solution (0.1 mg/ml water containing 2% acetone) was added and incubated at 22 °C for 20 min. The number of living cells was measured under a fluorescence microscope.

Results and Discussion

In our previous papers, orthovanadate, which is well known as an inhibitor of plasma membrane ATPases, was found to inhibit polyphosphoinositide (PI) metabolism and the active defense responses of pea tissue. From these results, we suggested that the plasma membrane ATPase may cross-talk with PI metabolism and may play an important role in signal transduction cascade leading to the active defense responses of pea tissues. As described in the introduction, however, orthovanadate was reported to mimic the effects of fungal elicitors in plant cultured cells. As a first step, therefore, the in vitro effect of orthovanadate on ATPase activity and PI metabolism in plasma membrane fraction, which was prepared from pea suspension cultured cells, was examined. As shown in Fig. 1, orthovanadate inhibited the ATPase activity in plasma membrane fraction from pea suspension cultured cells in a dose dependent manner as well as that from pea epicotyl tissues. One mM of orthovanadate completely negated the activity of plasma membrane ATPase of the cultured cells.

One mM of orthovanadate also inhibited above 30% of the incorporation of radioactivity from [γ-32P]ATP into PIP and PIP2, as compared to the water control in spite of the presence or absence of the elicitor from M. pinnata (Fig. 2). These results showed that orthovanadate interfered with enzymes such as phosphatidylinositol kinase (PI-kinase) and phosphatidylinositol-4-mono-
phosphate kinase (PIP-kinase) in PI metabolism. This result coincides with our previous data that orthovanadate inhibited the ATPase, PI-kinase and PIP-kinase in the plasma membranes prepared from pea epicotyl tissues. Thus, when taken together with our previous findings, it was shown that there is no difference between plasma membranes from suspension-cultured cells and those from epicotyl tissues in the sensitivity of these enzymes to orthovanadate. In other words, the *in vitro* effect of orthovanadate on the fundamental functions in plasma membranes of the cultured cells is as same as that of the tissues.

In the next step, the *in vivo* action of orthovanadate on the defense response in pea suspension-cultured cells was examined. As shown in Fig. 3, the elicitor from *M. piniodes* induced the accumulation of pea phytoalexin, pisatin, in the cultured cells as observed in pea epicotyl tissues. Copper(II) chloride, well known as an abiotic elicitor, also induced pisatin accumulation in a dose-dependent manner, while
3 mM of 

Orthovanadate also induced the accumulation of pisatin in a dose-dependent manner. These results indicate that orthovanadate acts as an abiotic elicitor on the suspension-cultured cells of pea as well as those of peanut and red bean. Thus, the effect of orthovanadate seems to be the same on the suspension-cultured cells of broad plant species. In addition, orthovanadate was reported to act as a non-specific suppressor for one of defense responses, the activation of endochitinase and β-1,3-glucanase, in the tissues of the four leguminous plant species tested.

It was reported that an endogenous elicitor was released from the kidney bean (Phaseolus vulgaris) cells which were partially damaged by infection, freezing or treatment with abiotic chemicals such as HgCl₂ and chloroform. Such an endogenous elicitor was thought to induce phytoalexin biosynthesis in the surrounding cells. We, therefore, examined the effects of orthovanadate, CuCl₂, and the elicitor from M. pinnate on the viability of pea suspension-cultured cells. As shown in Fig. 4, the elicitor (500 µg/ml), glucose equivalent from M. pinnate scarcely affected the viability of the cultured cells. On the other hand, viability of the cultured cells was decreased by above 100 µM of orthovanadate and above 30 µM of CuCl₂ in a dose-dependent manner. About 30% and 90% of the cells were damaged by treatment with 1 mM orthovanadate and 3 mM CuCl₂, respectively. These results suggested that the mode of action of these chemicals on pea suspension-cultured cells may be considerably different from that of the elicitor from M. pinnate. Thus, the induction of pisatin accumulation by orthovanadate and CuCl₂ seems to be due to their toxicity and it is thought that the damaged cells by treatment with these chemicals might release certain elicitor molecules to induce phytoalexin biosynthesis in the living cultured cells. This concept seems to be probable, because the production of pisatin looked like inversely proportional to the viability of the cells and also a highly toxic concentration of CuCl₂ could not apparently induce phytoalexin accumulation (Figs. 3 and 4).

In this connection, fluorescein diacetate has been used to detect in vivo either the plasma membrane's damage or the cytoplasmic pH. Yoshida et al. reported that the fluorescence in the cultured cells of mung bean was markedly reduced after chilling for 24 h, suggesting acidification of the cytoplasms. A decrease in
cytoplasmic pH was also induced by treatment of cultured cells with diverse biotic and abiotic elicitors. In *Ptosea hybrida* cultured cells, the primary processes during elicitation of the phenylpropanoid pathway was thought to be a change (inhibition) in the activity of the plasma membrane ATPase and a subsequent decrease in the proton gradient. In this case, orthovanadate was also an effective elicitor. Thus, together with the reports of Hattori and Ohba and Steffens et al., there is the possibility that the cytoplasmic acidification, which is induced by broad environmental stresses, switches on the signal transduction cascade leading to defense responses. Presently, it is unknown whether CoCl₂, orthovanadate or the elicitor from *M. pimodes* induces the cytoplasmic acidification in pea cultured cells and which plays a crucial role in phytoalexin production, cytoplasmic acidification or certain programmed cell death. However, since the elicitor from *M. pimodes* hardly induced any damage (fluorescence decrease) in pea cultured cells (Figs. 4; rapid cytoplasmic acidification might be more important for induction of defense responses in the cultured cells.

As described, the in vitro effect of orthovanadate on pea cultured cells is quite different from that on pea differentiated tissues. That is, orthovanadate acts as an elicitor or the cultured cells but as a suppressor on the differentiated tissues, while the chemical inhibited in vitro the ATPase and PI metabolism in plasma membrane fractions isolated from both cultured cells and tissues (Figs. 1 and 2; Togoda et al., Yoshimura et al.). Therefore, the action of orthovanadate only on the plasma membrane functions may fail to explain the mechanism of its apparently contradictory effect. We previously reported that plant cell wall may play important roles in recognition of pathogenic signals, determination of host-parasite specificity and regulation of defense responses. In fact, the generation of active oxygen species dependent on certain peroxidases(s) and the activity of ATPase in cell walls were non-specifically enhanced by the elicitor from *M. pimodes* and were inhibited by the suppressor in a species-specific manner.

The suppressor inhibited in vitro the ATPase activities in plasma membranes isolated from both the host and non-host plants of *M. pimodes*, while the ATPase activities of non-host cell walls were never inhibited in vitro by the suppressor, neither were those of cell walls isolated from non-hosts in vitro. Furthermore, it was found that orthovanadate and the suppressor, which were placed on the pea leaf surface, severely inhibited the ATPase activities associated with all membrane systems in pea epidermal cells. It is likely, therefore, that the cell wall-bound enzymes might affect or regulate in vivo the function of other organelles such as plasma membrane and vacuole. If so, the difference between the action of orthovanadate on the cultured cells and that on differentiated tissues seems to have resulted from the action of orthovanadate on the cell walls. Further experiments are needed to elucidate the mechanism of such a reversible effect of orthovanadate, however, we emphasize that the results and conclusions obtained from these experiments with cultured cells are not always applicable to the differentiated tissues of plants that are actually living in a real stressful environment.

References

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バナジン酸によるエンドウ培養細胞における
フィトアレキシン生産の誘導

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バナジン酸は、エンドウ組織に褐変病原エリシナーの処理で誘導される一連の防御反応を抑制することが報告されている。そこで、本研究ではバナジン酸のエンドウ培養細胞に対する影響を調べた。バナジン酸は培養細胞群分離した核変異誘導因子ホメオステシン代謝関連酵素や ATPase の活性を濃度依存的に阻害した。次に、褐変病原エリシナー、腐化剤(非生物的エリシナーの一種)、およびバナジン酸の in vitro での影響を調べたところ、いずれの条件処理においてもエンドウ培養細胞のミオチン産生を誘導した。褐変病原エリシナーはエンドウ培養細胞の増殖死（FDA の染色性喪失）を誘導しなかったが、腐化剤、バナジン酸は明かな毒性を示した。以上の結果とこれまでの知見に基づいて、エンドウ培養細胞に対しては非生物的エリシナーとして作用するバナジン酸の作用機構を考察した。