Signal Transduction against the Terpenoid-Stimulation in the Liverwort Cells

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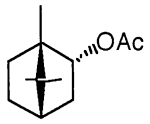
(Received July 28, 1997; CL-970590)

Addition of bornyl acetate to the cultured cells of *Marchantia polymorpha* caused a rapid increase of H_2O_2 and cAMP in the intracellular level. cAMP agonist (Sp-cAMPS) activated the H_2O_2 formation, while cAMP antagonist (Rp-cAMPS), protein kinase A inhibitor H-89, and Ca^{2+} ions inhibited the H_2O_2 formation.

Plants secrete various chemicals to protect themselves from other creatures.[1] For example, *Marchantia polymorpha* recognizes other plants as stress from the monoterpenoids secreted by them, and then, comes to produce phytoalexins which inhibit the growth of various higher plants.[2] Such phenomenon is broadly known from lower plants to higher plants.[3,4] Many studies were done about input (what kind of chemicals become stress for the plant?) and output (what kind of phytoalexins does the plant produce for its self-protection?). There are few studies about the mechanism of the defense reaction against chemical stress. Previously, we showed that cultured cells of *M. polymorpha* act a defense reaction against monoterpenoids, especially bornyl acetate (**1**), with the transient formation of active oxygen species, mainly hydrogen peroxide.[2,5] What we wish to show in this paper is the dependence of H_2O_2 formation on intracellular cAMP and on protein kinase A activity.



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In many cases, a signal transduces via a G protein(s). To clarify the participation of the G protein(s) in the signal transaction of bornyl acetate stimulation, the effect of nonhydrolyzable analog, guanosine 5'-(3-*O*-thio)triphosphate (GTPγS),[6] against the formation of H_2O_2 was examined.[7] As shown in Table 1, H_2O_2 was slightly formed in the presence of 200 μM GTPγS alone, whereas in the presence of GTPγS plus bornyl acetate (**1**), H_2O_2 formation was increased up to 2-fold against the case of bornyl acetate treatment only. This shows that the action of bornyl acetate (**1**) on H_2O_2 formation may involve a G protein(s), which has been shown for a wide variety of animal and higher plants.

One reasonable pathway for the formation of H_2O_2 against the bornyl acetate stimulation is that a signal via a G protein leads to Ca^{2+} influx, which then activates a Ca^{2+}-dependent protein kinase and ultimately the H_2O_2-generating enzyme system. However, the formation of H_2O_2 stimulated by 4 mM of (-)-bornyl acetate (**1**) in the cultured cells of *M. polymorpha* was diminished when 10 mM of Ca^{2+} was added in the medium. On the other hand, when the cells were washed three times in Ca^{2+}-free medium containing 1 mM EDTA, a significant increase in the formation of H_2O_2 was observed. The antibiotic ionophore A 23187,[10] which is widely used to study the regulatory role of Ca^{2+} in biological systems did not enhance the formation of H_2O_2. In the case of the higher plant-pathogen system, it is thought that Ca^{2+} participates in initiating the higher plant defense reactions.[11,12] However, in the case of the defense reactions of liverworts against the bornyl acetate stimulation, Ca^{2+} does not participate in the formation of H_2O_2, or if it participates, Ca^{2+} plays a role as a messenger of off-response against the bornyl acetate stimulation.

Table 1. Effect of activator and inhibitor on H_2O_2 release from the cultured cells of *M. polymorpha*

Treatment	Relative H_2O_2 concentration (%)
4 mM Bornyl acetate	100
200 μM GTPγS	3
200 μM GTPγS + 4 mM Bornyl acetate	209
10 mM Ca^{2+} + 4 mM Bornyl acetate	9
1 mM EDTA + 4 mM Bornyl acetate	179
5 μM Ionophore A 23187	11
200 μM Rp-cAMPS + 4 mM Bornyl acetate	158
200 μM Sp-cAMPS + 4 mM Bornyl acetate	66
100 nM H-89 + 4 mM Bornyl acetate	51

Another reasonable pathway for the formation of H_2O_2 is that a signal via a G protein leads to cAMP influx and then activates a cAMP-dependent protein kinase and ultimately the H_2O_2-generating enzyme system. The change in intracellular cAMP level in the cultured cells of *M. polymorpha* was determined[13] by the method as described in refs. 15 and 16. Figure 1 shows that intracellular cAMP increased after the addition of bornyl acetate (**1**). The concentration of cAMP

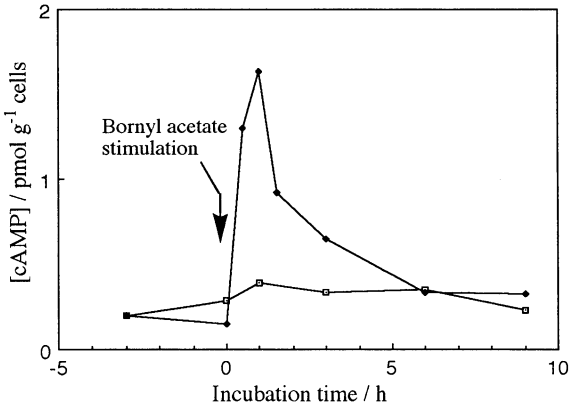

Figure 1. Time course in the intracellular cAMP level in the cultured cells of *M. polymorpha*. The content of cAMP in the cells was determined by radioimmunoassay (—◆—). For the control intracellular cAMP level in the cultured cells without bornyl acetate stimulation was also monitored (—□—).

reached a maximum value at 1-2 h incubation. In a control culture without the addition of bornyl acetate (**1**), the change in cAMP content was negligible. These results suggest that cAMP plays significant roles in the defense process involving the formation of H_2O_2 in the cultured cells of *M. polymorpha*.

To evaluate the role of cAMP-dependent protein kinase (protein kinase A) in the signal transduction against the bornyl acetate stimulation, we examined the effect of Sp-diastereoisomer of cyclic adenosine-3',5'-monophosphothioate (Sp-cAMPS) and Rp-isomer on the H_2O_2 formation in the cultured cells of *M. polymorpha*. Sp-cAMPS, which is a cAMP agonist,[17] activated the H_2O_2 formation, whereas Rp-isomer inhibited the formation of H_2O_2. It has been reported that Rp-cAMPS is a cAMP antagonist; it binds to the regulatory subunit of protein kinase A.[18,19] On the other hand, protein kinase A inhibitor H-89[20] also inhibited the formation of H_2O_2, as shown in Table 1. These results obtained suggest that the activation of the protein kinase A is essential for the signal transduction against the bornyl acetate stimulation.

From above mentioned results, we suggest the following model for the signal transduction against the terpenoid-stimulation in the liverwort cells. When the terpenoid binds to a receptor localized in the cell surface of the liverwort, the receptor associates with a G protein, and then the G protein transduces the signal, triggering the activity of an adenylate cyclase. cAMP which is formed by adenylate cyclase stimulates a protein kinase A. The protein kinase A promotes the generation of active oxygen species by phosphorylating an intracellular protein(s). This signal transduction resembles more the olfactory transduction in animals than the signal transduction against elicitors in higher plants, such as that occurring in a higher plant-pathogen system. Since it is known that the activation of the protein kinase A causes the repression of the generation of active oxygen species in phagocytic cells,[21,22] in the case of liverworts the active oxygen species generation may depend on the activation of the protein kinase A, but further works are necessary to clarify the protein(s) phosphorylated by protein kinase A.

The present work was in part supported by Grant-in-Aids for Scientific Research No. 09480142 from the Ministry of Education, Science and Culture of Japan.

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13 After bornyl acetate (**1**) was added, an aliquot of the cell suspension (2 ml) was removed at a regular time interval and mixed with 200 ml of 50 % cold trichloroacetic acid (TCA). The insoluble material was removed by centrifugation and then the TCA was removed with diethyl ether. The water phase was lyophilized and then the amounts of cAMP in the dried sample were determined with a radioimmumoassay technique using Yamasa cAMP test kit.[14]
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