Original Article

Transmembrane Signalling Operated at Rice Blade Cells Stimulated by Blast Fungus Elicitor II. Participation of Calcium Modulated Protein^{*1}

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The effects of molecular probes on the dynamic behaviours of O_2^- generation and α -linolenate release from rice (*Oryza sativa*) blade tissue preparations with press-injured spot (2 mm), pre-treated with the molecular probes and post-stimulated with blast fungus (*Pyricularia oryzae*) elicitor, were further surveyed. Compound W-7 or ophiobolin A, potent inhibitors on the function of CaM (calmodulin), strongly inhibited both O_2^- generation at 10 μ M and α -linolenate release at 500 μ M after the elicitor stimulation. The comparative coupled-enzymic analysis on the behaviour of free CaM in the healthy or infected rice blades with blast fungus conidia revealed an immediate decrease of free CaM by the infection. TPA (10 μ M), an agonist of diacylglycerol for protein kinase C, did not activate both parameters with the elicitor stimulation, although TPA (10 μ M) markedly activated the α -linolenate release at the later phase with the stimulation. The application of H-7 (1 mM) or staurosporin (10 μ M), unspecific inhibitors on protein kinases, inhibited the O_2^- generation, but staurosporin (50 μ M) activated the α -linolenate release at the later phase and H-7 (1 mM) inhibited the α -linolenate release after the elicitor stimulation. The application of 1,2-benzisothiazol-3(2H)-one 1,1-dioxide (1 mM) with the elicitor stimulation further activated the O_2^- generation and markedly activated the α -linolenate release at the later phase. The lines of evidence strongly bore out the suggestion that the formations of Ca^{2+} -MPs (Ca^{2+} -calcium modulated proteins), which signal-coupled with the operation of phospholipase C system, play an indispensable role in activating rice blade O_2^- forming redox system and phospholipase A_2 .

INTRODUCTION

In the previous paper,¹⁾ it was postulated that the signal-activated phospholipase C system as a transmembrane signalling system

is operating at rice (*Oryza sativa*) blade cells stimulated by blast fungus (*Pyricularia oryzae*) elicitor, and that the O_2^- generation and α -linolenate release, the two earlier biochemical events activated by the elicitor stimulation, reveal a strong dependency on the mobilization of Ca^{2+} from Ca^{2+} stores by IP_3 (inositol 1,4,5-trisphosphate). It has been established in animal cells that diacylglycerol, one of the products from plasma membrane PIP_2 (phosphatidylinositol 4,5-bisphosphate) by the signal-activated phospholipase C, plays an indispensable role in activating protein kinase C as an agonist of Ca^{2+} and that the phosphorylation of proteinic regulatory factors by protein kinase

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C activates O_2^- forming redox system and phospholipase A_2 .²⁻⁵⁾ In this study, we attempted to survey the relationships between Ca^{2+} as a second messenger and the two parameters, O_2^- generation and α -linolenate release from rice blade tissue stimulated by blast fungus elicitor, using several molecular probes as in the previous paper.¹⁾

MATERIALS AND METHODS

1. Reagents

W-7; N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide HCl, ophiobolin A and staurosporine were purchased from Sigma. TPA; 12-O-tetradecanoylphorbol-13-acetate, was purchased from LC Services Co./Funakoshi Chemical Co., Ltd. (Tokyo). H-7; 1-(5-isoquinolinesulfonyl)-2-methylpiperazine 2HCl was purchased from Seikagaku Kogyo Corp. (Tokyo). CaM (calmodulin) dependent phosphodiesterase (bovine heart muscle), alkaline phosphatase (calf intestine), adenosine deaminase (calf intestine), authentic CaM (porcine brain) and cyclic adenosine-3',5'-monophosphate were purchased from Boehringer Mannheim Yamanouchi Co., Ltd. (Tokyo). SAC·Na (1,2-benzisothiazol-3(2H)-one 1,1-dioxide sodium salt) was obtained from Wako-junyaku Co., Ltd. (Tokyo). Other reagents used were the highest grade commercially available.

2. Plant Material and Blast Fungus Elicitor

Those were the same as described in the previous paper.¹⁾

3. Application of Chemicals, Stimulation by Blast Fungus Elicitor and Determination of O_2^- Generation and α -Linolenate Release

Those were also the same as described in the previous paper.¹⁾ The aqueous solution of staurosporine was prepared by diluting 20 mM staurosporine dimethylsulfoxide solution with deionized water. The application of 0.5% dimethylsulfoxide aqueous solution (5 μ l) onto the 2 mm press-injured spot did not interfere with the both parameters. The aqueous solution of TPA was prepared by diluting 10 mM TPA ethanol solution with deionized water. The aqueous solution of ophiobolin A was prepared by diluting 50 mM ophiobolin A

ethanol solution with deionized water. The application of 1% ethanol aqueous solution (5 μ l) onto a 2 mm press-injured spot did not interfere with the both parameters.

4. Determination of CaM in Rice Blade

Rice seedlings at four-leaf stage were inoculated by spraying of blast fungus conidial suspension (3×10^5 conidia/ml) for the infected run, and were sprayed with sterile water for control run. The sample was respectively taken in time-course schedule. The total CaM was extracted in the presence of 5 mM EDTA and 5 mM EGTA to dissociate complexes of Ca^{2+} -CaM and proteins using 50 mM Tris-HCl buffer solution (pH 7.5) under homogenizing with Biotron (BT 10/20-3500, Switzerland). The free CaM was extracted in the absence of EDTA and EGTA using the same buffer under homogenizing. After the removal of accompanied proteins by treatment with 5% trichloroacetic acid⁶⁾ and Bond Elut DEAE cartridge (Analtech International Co.), CaM was quantitatively determined by coupled-enzymic assay using CaM dependent phosphodiesterase (bovine heart muscle).⁷⁾ The coupled-enzymic assay system consisted of three species of enzymes, CaM dependent phosphodiesterase, alkaline phosphatase and adenosine deaminase. And the decrease of absorbance at 265 nm, which corresponded to the decrease of adenosine, was consistent with phosphodiesterase activity depending on the amount of CaM. The amount of rice blade CaM, equal to 1 μ g of the authentic CaM, was defined to 1 unit.

RESULTS

1. Effects of W-7 and Ophiobolin A on Both of the Parameters

The effects of W-7 or ophiobolin A, inhibitors on the function of CaM,⁸⁻¹⁰⁾ on both the parameters, O_2^- generation and α -linolenate release, are respectively shown in Figs. 1 and 2. It was observed that W-7 (10 μ M) and ophiobolin A (10 μ M) strongly inhibited the O_2^- generation after the elicitor stimulation and that W-7 (500 μ M) and ophiobolin A (500 μ M) inhibited the α -linolenate release after the elicitor stimulation. It was noted that the signal-coupled activation of O_2^- forming redox

Fig. 1 Effects of W-7 on O_2^- generation and α -linolenate release from 2 mm press-injured rice blade tissue stimulated by blast fungus elicitor.

O_2^- FS activity; activity of O_2^- forming redox system in terms of O.D.₅₆₀ nm $\cdot$ ml $^{-1}$ ·min $^{-1}$ ·mg fresh weight $^{-1}$. PLA₂ activity; activity of phospholipase A₂ in terms of relative fluorescence intensity at 540 nm·min $^{-1}$ ·mg fresh weight $^{-1}$. Refer to the previous report¹⁾ regarding the details of determinations on two parameters and control runs without the application of chemical. ○: 2 mm press-injury with application of 5 μ l of 10 μ M or 500 μ M W-7 (control), ●: application of W-7 and additional stimulation by blast fungus elicitor. The arrow indicates the time of stimulation. The similar patterns were observed in duplicated measurements.

system was more sensitive to both W-7 and ophiobolin A than the activation of phospholipase A₂.

2. Dynamic Behaviour of CaM in Rice Blade Tissue Infected by Blast Fungus Conidia

The content of total CaM in healthy or infected rice blades was approximately 0.4 units/g fresh weight, and did not respectively undergo an appreciable change by the infection in time-course experiments (data not shown). It seemed likely that the contents of

Fig. 2 Effects of ophiobolin A on O_2^- generation and α -linolenate release from the press-injured rice blade tissue.

○: 2 mm press-injury with application of 5 μ l of 10 μ M or 500 μ M ophiobolin A (control), ●: application of ophiobolin A and additional stimulation by blast fungus elicitor. The arrow indicates the time of stimulation. The similar patterns were also observed in duplicated measurements.

total CaM in rice blade tissue might be lesser than in other plants and animal tissues. It has been reported that the content is about 4 units/g in spinach leaf tissue¹¹⁾ and 400–600 units/g in rat brain tissue.¹²⁾ The dynamic behaviour of free CaM in healthy or infected rice blade with a compatible race (HOKU 373)¹⁾ is shown in Fig. 3. It was observed that the behaviour of free CaM in healthy rice blades might reveal a pattern in normal growth and that the behaviour of free CaM in infected rice blade revealed the immediate decrease of free CaM down to about 0.05 units/g fresh weight 1 day after spray inoculation of blast fungus conidia.

3. Effects of TPA on Both of the Parameters

The effects of TPA, potent activator on

Fig. 3 Dynamic behaviour of free CaM in rice blade tissue inoculated with blast fungus conidia. The conidia of compatible blast fungus race (HOKU 373) was inoculated by usual spray-method. As for coupled enzymatic analysis of CaM, see MATERIALS AND METHODS. ○: healthy run, ●: infected run. The similar behaviours were observed in duplicated experiments.

Fig. 4 Effects of TPA on O_2^- generation and α -linolenate release from the press-injured rice blade tissue.

○: 2 mm press-injury with application of $5 \mu\text{l}$ of $10 \mu\text{M}$ TPA (control), ●: application of TPA and additional stimulation by blast fungus elicitor. The arrow indicates the time of stimulation. The similar patterns were observed in duplicated measurements.

protein kinase C,¹³⁻¹⁵⁾ on both the parameters are shown in Fig. 4. It was observed that TPA ($10 \mu\text{M}$) did not activate the O_2^- generation even after elicitor stimulation and that TPA ($10 \mu\text{M}$) inhibited α -linolenate release at the earlier phase but markedly activated α -linolenate release at the later phase with elicitor stimulation.

4. Effects of H-7 and Staurosporine on Both of the Parameters

The effects of H-7 or staurosporine, unspecific inhibitors on protein kinases,¹⁶⁻¹⁹⁾ on both the parameters are respectively shown in Figs. 5 and 6. It was observed that H-7 (1 mM) and staurosporine ($100 \mu\text{M}$) inhibited O_2^- generation after elicitor stimulation, that staurosporine ($50 \mu\text{M}$) rather activated α -linolenate release at the later phase after elicitor stimula-

Fig. 5 Effects of H-7 on O_2^- generation and α -linolenate release from the press-injured rice blade tissue.

○: 2 mm press-injury with application of $5 \mu\text{l}$ of 1 mM H-7 (control), ●: application of H-7 and additional stimulation by blast fungus elicitor. The arrow indicates the time of stimulation. The similar patterns were observed in duplicated measurements.

Fig. 6 Effects of staurosporine on O_2^- generation and α -linolenate release from the press-injured rice blade tissue.

○: 2 mm press-injury with application of 5 μ l of 100 μ M or 50 μ M staurosporine (control), ●: application of staurosporine and additional stimulation by blast fungus elicitor. The arrow indicates the time of stimulation. The similar patterns were also observed in duplicated measurements.

Fig. 7 Effects of SAC·Na on O_2^- generation and α -linolenate release from the press-injured rice blade tissue.

○: 2 mm press-injury with application of 5 μ l of 1 mM SAC·Na (control), ●: application of SAC·Na and additional stimulation. The similar patterns were observed in duplicated measurements.

tion, and that H-7 (1 mM) inhibited α -linolenate release after elicitor stimulation.

5. Effects of SAC·Na on Both of the Parameters

The effects of SAC·Na, an activator on the O_2^- generation from rice blade tissue infected by blast fungus,^{20,21)} on both the parameters are shown in Fig. 7. It was observed that SAC·Na (1 mM) alone gave an moderate activation on O_2^- generation at earlier phase when applied to the press-injured spots, and that elicitor stimulation further activated and shifted the O_2^- generation towards the earlier phase. It was also observed that SAC·Na (1 mM) alone might slightly activated that α -linolenate release, and that the elicitor stimulation markedly activated the release at the

later phase (Fig. 1A and 1B in Part XII¹⁾).

DISCUSSION

Using press-injured rice blade tissue stimulated by purified blast fungus elicitor,¹⁾ the relationships between Ca^{2+} as the second messenger and the activation of two parameters, the O_2^- generation and α -linolenate release, were surveyed.

Both W-7 (10 μ M) and ophiobolin A (10 μ M), potent inhibitors on a function of CaM,⁸⁻¹⁰⁾ strongly inhibited the signal-coupled O_2^- generation (Figs. 1 and 2). The free CaM (0.03 units/g fresh weight; 7.5% of total CaM) of rice blade tissue decreased 1 day after spray inoculation of blast fungus conidia (Fig. 3). This revealed that free CaM was mobilized to form Ca^{2+} -CaM by blast fungus infection. Both W-7 (500 μ M) and ophiobolin A (500 μ M) also strongly inhibited the signal-coupled α -

linolenate release (Figs. 1 and 2). The presence of plant CaM²²⁾ and Ca²⁺-CaM dependent protein kinase linked with the activation of phospholipase in potato leaves have been reported.²³⁾ The lines of evidence indicated that both activation of O₂⁻ forming redox system and phospholipase A₂ in elicitor-stimulated rice blade cells are strongly dependent on the formation of Ca²⁺-MP (Ca²⁺-calcium modulated protein), which includes Ca²⁺-CaM and/or Ca²⁺-CDPK (Ca²⁺ dependent protein kinase).²⁴⁾

TPA (10 μ M) potent activator on protein kinase C,¹³⁻¹⁵⁾ inhibited both the O₂⁻ generation and α -linolenate release at earlier phase after elicitor stimulation (Fig. 4), suggesting that protein kinase C might not participate in the activation of rice blade O₂⁻ forming redox system and phospholipase A₂ at earlier phase after elicitor stimulation. And both H-7 (1 mM) and staurosporine (100 μ M), unspecific inhibitors on protein kinase,¹⁶⁻¹⁹⁾ inhibited the O₂⁻ generation. And H-7 (1 mM) markedly inhibited α -linolenate release, and staurosporine (50 μ M) moderately inhibited α -linolenate release at earlier phase implying that the phosphorylation reactions might be involved in the activation of O₂⁻ forming redox system and phospholipase A₂ (Figs. 5 and 6). The lines of evidence revealed that both the activations of O₂⁻ forming redox system and phospholipase A₂ of elicitor-stimulated rice blade cells are strongly dependent on Ca²⁺-CaM dependent protein kinase and/or Ca²⁺-CDPK.^{23,24)} It has been reported that the activity of *Dunaliella*-CDPK is inhibited by relatively higher doses of CaM-inhibitors²⁵⁾ and that soybean-CDPK has a regulatory domain which is 40% identical to plant calmodulin.^{26,27)}

It was noted in this study that elicitor-stimulated activation of phospholipase A₂ in rice blade cells was relatively insensitive to Ca²⁺ channel blockers (Fig. 7 in Part XII¹⁾) and also to inhibitors on the formation of Ca²⁺-CaM (Figs. 1 and 2). It has been known that the formation of Ca²⁺-CDPK is relatively insensitive to inhibitors on the formation of Ca²⁺-CaM.²⁵⁾ This bears out a hypothesis that the signal transduction system from opening of Ca²⁺ channel to activation of phospholipase A₂ might utilize a molecular

species of Ca²⁺-CDPK. As previously reported,¹⁾ the presence of Ca²⁺ channel in rice blade cells, that was relatively insensitive to three blockers, was also indicated (Fig. 7 in Part XII¹⁾). Further studies are required to elucidate the presence and characteristics of Ca²⁺ channels and Ca²⁺-MPs participating in the coupling between the transmembrane signalling system and two intracellular signalling systems, which are thought to individually activate the two parameters used in this study, O₂⁻ generation and phospholipase A₂.

It was also noted in this study that TPA (10 μ M), an agonist of diacylglycerol, markedly activated the α -linolenate release at the later phase after the elicitor stimulation (Fig. 4). The similar tendencies to the later activation were observed by the application of neomycin (1 mM),¹⁾ an inhibitor on PI (phosphatidylinositol) turnover, or staurosporine (50 μ M), an inhibitor on protein kinases (Fig. 6). Interestingly, the single applications of these three molecular probes also gave similar tendencies to the later activation (Fig. 2 in Part XII¹⁾ and Figs. 4 and 6 in this report), although the correct explanation of these phenomena is not available at present. It has been reported that the activities of PI kinase and PIP (phosphatidylinositol 4-phosphate) kinase at plasma membrane of tobacco suspension culture cells are regulated by a negative feedback inhibition due to the increasing of cytosolic Ca²⁺.²⁸⁾ It has been reported that the signal-coupled activation of phospholipase C in animal cells is also indirectly regulated by a negative feedback inhibition due to the increase of diacylglycerol,^{13,15)} and that the presence of alternatively signal-activated phospholipase A₂ system, which was positively regulated by the increase of diacylglycerol, is postulated.^{29,30)} When the presence of similar signal-activated phospholipase A₂ system in rice blade cells, which was positively regulated by the negative feedback inhibition on PI turnover is hypothesized, the activation of phospholipase A₂ at later phase without the operation of signal-activated phospholipase C system could be explainable, but a detail analysis concerned remains for future exploration.

SAC·Na (1 mM) enhanced both the activa-

tions of O_2^- forming redox system and phospholipase A_2 (Fig. 7). Probenazole, 3-allyloxy-1,2-benzisothiazole 1,1-dioxide, is a prodrug of SAC, and is practically used as a priming effector to give a state of long-lasting systemic acquired-resistance against rice blast disease.³¹⁾ It is noted that SAC·Na activates O_2^- forming redox system at very early phase and that it activates the rice blade phospholipase A_2 at the later phase after elicitor stimulation. This implies that the activation of phospholipase A_2 at later phase is effective for the establishment of systemic acquired-resistance. The lines of evidence suggest that an action site of SAC exists within the transmembrane signalling and intracellular signalling systems to activate the two parameters and that the systemic distribution of SAC in rice blade cells give a state of systemic acquired resistance enhancing the signal-coupled activations of O_2^- forming redox system and phospholipase A_2 , which are subsequently linked to induction of a whole set of effective resistant reactions, immediately after blast fungus infection.^{1,20)} Very recently, it has been reported that a novel compound, *N*-cyanomethyl-2-chloroisonicotinamide (NCI), has the similar priming effect on probenazole enhancing the induction of lipoxygenase and peroxidase, when rice seedlings are treated with NCI *via* root system.³²⁾ Both chemical structures of NCI and SAC²⁰⁾ have common functional moieties, carbonylimide with individual electron drawing group such as 2-chloropyridinyl or sulfonyl group. An interesting evidence, which was obtained using NCI, is that NCI clearly activated PI turnover in elicitor-stimulated rice-embryo culture cells.³³⁾ This evidence and lines of evidence regarding signal transduction system of rice blade cells, which were described in the previous¹⁾ and this report, bear out the suggestion that an action-site of priming effectors like NCI and SAC, to give a state of long-lasting systemic acquired resistance, duly resides in the signal-activated phospholipase C system, although the detail regulatory mechanisms as for the activation of phospholipase C are still a matter of controversy related to a signal-activated PIP kinase system and/or inhibitory constraint factor of phospholipase C.^{34,35)}

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要 約

イネいもち病菌エリシターで刺激したイネ葉身細胞において作動する膜情報伝達系. II. カルシウム調節タンパク質の関与

加納大聖, 芳賀 実, 関沢泰治

前報に続き、イネ葉身細胞の膜情報伝達系に共役する O_2^- 生成酸素系およびホスホリパーゼ A_2 (本研究における二つの指標) の活性化反応に係わる細胞内情報伝達系を探索した。分子プローブとして W-7, オフィオポリン A, TPA, H-7, およびスタウロスポリンなどを用い、前報と同様の計画の下で探索を行ない、 Ca^{2+} 調節タンパク質 (CaM あるいは CDPK) が両指標の活性化反応に重要な役割を果たすこと、プロテインキナーゼが関与するなら Ca^{2+} -CaM 依存性プロテインキナーゼあるいは Ca^{2+} CDPK によるものと推定した。なお、ホスホリパーゼ A_2 の活性化反応の制御には未知の制御経路が介在している可能性を推考した。1,2-ベンツイソチアゾール-3(2H)-オン 1,1-ジオキシドは早期相において O_2^- 生成を高進させ、 α -リノレン酸放出を後期相で著しく高進させた。プロベナゾールがプライミング効果剤として付与する全身獲得抵抗性での両指標が、エリシター刺激のみによる型と異なることが注目された。同様の機能を有する NCI は PI ターンオーバーを加速することが最近報告されたので、前報および本報による知見とともに、総合考察し、この種の非殺菌性防除剤の作用部位はイネ葉身細胞の膜情報伝達系 (ホスホリパーゼ C 系) に存在すると推定された。

* イネの誘導防御機構における反応カスケードの因果律に関する研究 (第13報)