Plasma Membrane Perturbation in Association with Calcium Ion Movement Followed by Fungal Elicitor-stimulated Oxidative Burst and Defense Gene Activation in Potato Tuber

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Abstract

Chlortetracycline (CTC) is known to fluoresce when interacting with Ca²⁺ in hydrophobic environments such as membranes. CTC fluorescence in potato tubers was rapidly reduced by hyphal wall component-elicitor (HWC-elicitor) from Phytophthora infestans in a dose-dependent manner, suggesting that Ca²⁺ in the plasma membrane was released by the elicitor. Pre-treatments of the tuber slices with verapamil and EGTA, a Ca²⁺ channel blocker and an extracellular Ca²⁺ chelator, respectively, suppressed the reduction of CTC fluorescence induced by the HWC-elicitor. The reduced fluorescence was preceded by an oxidative burst observed in the tuber tissues loaded with luminol after stimulation with HWC-elicitor. Application with HWC-elicitor also immediately reduced fluorescence of the CTC-loaded, Ca²⁺-bound plasma membrane fraction in vitro. Moreover, RNA gel blot analysis showed that verapamil and EGTA dramatically suppressed the accumulation of transcript for phenylalanine ammonia-lyase induced by the elicitor. These results demonstrate that extracellular Ca²⁺ is rapidly released from the plasma membrane by the treatment with HWC-elicitor and plays a key role in signal transduction leading to the oxidative burst and the activation of a defense gene.

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Key words: calcium ion, chlortetracycline, defense gene, oxidative burst, Phytophthora infestans, potato tuber.

INTRODUCTION

The oxidative burst has been suggested to be a primary event responsible for triggering the cascade of defense response in various plant species against infection with avirulent pathogens or treatment with pathogen-derived elicitors²,³,⁶,⁸⁻¹⁰,²⁰,²⁵,²⁶,²⁸. Activation of an O₂⁻-generating system in the plasma membrane of plant cells is thought to contribute to an oxidative burst within a few minutes after stimulation with elicitors²⁻¹¹. Since the extracellular Ca²⁺ chelator ethyleneglycolbis(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA) inhibits the elicitor-stimulated oxidative burst as well as the hypersensitive reaction in plant tissues²⁹, protoplasts³⁰ or suspension cultured cells²³,²⁵,³⁰, involvement of Ca²⁺ in the oxidative burst has been postulated. Recently, an NADPH-dependent O₂⁻-generating reaction was shown to be activated by an elicitor treatment in an in vitro system consisting of Ca²⁺-bound plasma membrane-rich fraction and cytosolic protein fraction of potato tuber tissues, ATP and NADPH³¹. The in vitro activation of the O₂⁻-generating reaction was abolished by the pre-treatment with EGTA³¹. In contrast, a continuously activated NADPH-dependent O₂⁻-generating reaction in plasma membrane fraction isolated from hypersensitively reacting potato tuber tissues was not dependent on Ca²⁺, because administration of EGTA did not inhibit the activity³¹. These lines of evidence suggest that Ca²⁺ is required for the activation process of the NADPH-dependent O₂⁻-generating system in plant cells.

An influx of extracellular Ca²⁺ into the cytoplasms of elicitor-treated cells is assumed to be responsible for the activation of O₂⁻-generating system in the plasma membrane. An elicitor-stimulated Ca²⁺ influx into plant cells or an increase in intracellular free Ca²⁺ has been supported by the incorporation of extracellular "Ca²⁺ into elicitor-treated cells²³,³¹" by an increase in fluorescence of Fluo-3 loaded cells²¹, and by the use of aequorin-transgenic cells¹⁹,²⁰ following treatment with elicitors. We also recently showed that Ca²⁺-channel blockers effectively inhibited the elicitor-stimulated
oxidative burst\textsuperscript{23}.

This report investigates elicitor-stimulated influx of extracellular Ca\textsuperscript{2+} and its association with the rapid oxidative burst. The antibiotic chlorotetracycline (CTC) exhibits specific fluorescence for Ca\textsuperscript{2+} (Em max at 514 nm with excitation at 401 nm) interacting with the Ca\textsuperscript{2+} in a hydrophobic environment such as membranes in animal cells, but not with free Ca\textsuperscript{2+} \textsuperscript{4,30}. This fluorescent probe, CTC, is a useful tool for the sequential chasing of the membrane-bound Ca\textsuperscript{2+} in plant cells by continuously monitoring changes in CTC fluorescence. We report here that extracellular Ca\textsuperscript{2+} is rapidly released from the plasma membrane after treatment with the HWC-elicitor and serves as an important signal for the oxidative burst and the activation of a defense gene involved in antimicrobial responses such as lignin or salicylic acid synthesis.

**MATERIALS AND METHODS**

**Plant and fungal materials** Tubers of potato cv. Rishiri (an interspecific hybrid between *Solanum tuberosum* L. and *S. demissum* with R1 resistance gene against *Phytophthora infestans*) was used for the preparation of tuber slices (2 mm thick), and the plasma membrane-rich fraction. The tuber slices from tuber parenchyma tissues were prepared with a microtome, rinsed with distilled water and incubated on wet filter paper in a sealed plastic chamber at 20±1°C in the dark for 18 hr. For total RNA preparation, the tuber discs were aged for 23 hr. *Phytophthora infestans* (Mont.) de Bary (race 0) was used for the preparation of hyphal wall components elicitor (HWC-elicitor). HWC-elicitor was prepared from fungal mycelial mats grown on liquid medium according to the method as described before\textsuperscript{12}).

**Preparation of plasma membrane fraction and Ca\textsuperscript{2+}-bound plasma membrane fraction** A plasma membrane-rich fraction was prepared from aged potato tuber discs in the presence of EGTA at 10 mM according to the modified methods of Yoshida et al.\textsuperscript{30} as previously reported\textsuperscript{4}. Preparation of the Ca\textsuperscript{2+}-bound plasma membrane-rich fraction was as follows: the plasma membrane-rich fraction was initially partitioned with two layers of aqueous polymer, precipitated by centrifugation at 100,000×g for 90 min, resuspended with 2 mM CaCl\textsubscript{2} in 0.1 M K-phosphate buffer (pH 7.4), centrifuged at 100,000×g for 90 min and finally suspended in 0.1 M K-phosphate buffer (pH 7.4).

**CTC-loading and monitoring fluorescence intensity** The entire surface of aged tuber discs was treated with 10 μl of CTC-solution. Fluorescence intensity, fluorescence spectra and differential fluorescence spectra of CTC-loaded tuber discs and of the plasma membrane-rich fraction were determined using a fluorescence spectrophotometer (Model 805, Hitachi Co., Ltd.). The fluorescence from the discs was monitored by using a sample holder (part No 650-0161) for determination of the surface fluorescence of solid materials.

The fluorescence of plasma membrane-rich fraction was monitored at 20°C using a non-fluorescing quartz cell (10×10 mm) containing a small magnetic stirrer. The monitoring conditions for CTC fluorescence were set as follows: band pass, 5 nm for both excitation and emission; response, 2 sec; EM filter, 430 nm (this was used only in the case of tissue discs); time scan, 0.5 cm/min; excitation, 401 nm; and emission, 514 nm.

**Detection of oxidative burst** Generation of hydrogen peroxide was continuously monitored by measuring chemiluminescence following treatment of aged tuber slices (1.8×0.9 cm, 2 mm thick) with a mixture of HWC-elicitor (1 mg/ml) and luminol (2.5 mM) in 10 mM Tris-HCl buffer (pH 7.4). The mixture was applied to the tissue surface by injecting a space between a thin transparent wrap film that covered tuber tissue and the surface. Chemiluminescence from the treated surface through the film was measured at 425 nm without any excitation in the dark by using the photon counting mode of a biochemiluminescence spectrophotometer (Hitachi Co., Ltd.). Photon counting conditions were the following: emission, 425 nm; band path, 5 nm; gate time, 20 sec; time average, 20 sec; temperature, 20±1°C.

**RNA gel blot analysis** After an aging period of 23 hr at 20°C, tuber discs, 2.0 cm in diameter and 2 mm-thick, were pre-treated for 1 hr with 100 μl of EGTA (1 mM), verapamil (100 μM) or water, and then the solutions were wiped off. A mixture of 100 μl of the HWC-elicitor (1 mg/ml, final concentration) and one of the inhibitors was applied to the cut surface of tuber discs in the dark. RNA from potato tuber discs was prepared as described by Yoshioka et al.\textsuperscript{30}. Ten micrograms of RNA were separated by electrophoresis on a 1.0% agarose-formaldehyde gel. Amounts of RNA in the gel after electrophoresis were monitored for consistency among treatments by fluorescence of acridine orange (0.5 μg/ml)-stained RNA under UV light. The separated RNA was transferred to hybond-N\textsuperscript{+} membrane (Amersham, Aylesbury, UK). A 1.2-kb fragment of potato PAL-1 cDNA was used as a probe\textsuperscript{46}. The cDNA was labeled with α-32P dCTP using a random-primed DNA labeling kit (Takara Inc., Kyoto Japan). Hybridization and washing were performed as described previously\textsuperscript{38}.

**RESULTS**

**Optimum conditions for CTC loading on the potato tuber discs**

It was reported that CTC also interacts with Mg\textsuperscript{2+} in the hydrophobic environment (Em max at 530 nm with excitation at 401 nm)\textsuperscript{4,30}. However, analyses of differential emission spectra between CTC-loaded and non-loaded plant systems showed almost symmetrical emission spectra with a maximum at 514 nm (excitation at 401 nm). These results indicate that the fluorescence of CTC-loaded plant systems is derived predominantly.
Fig. 1. Profiles of the fluorescence emission from the surface of aged tuber discs (20 mm in diameter) on which CTC (10 μl) was applied at various concentrations as designated (mM). Emission was continuously monitored at 514 nm with excitation at 401 nm after application of CTC. In the case of loading at 1 mM, an additional application of 1 mM CTC was done 10 min after initial CTC-loading (1).

Fig. 2. Time-course changes in CTC fluorescence from aged tuber discs after application of HWC-elicitor. CTC (1 mM) was applied to the surface of the discs (10 mm in diameter) for 10 min, rinsed with distilled water, and then HWC-elicitor was applied at various concentrations (μg/ml) as designated.

Fig. 3. Effects of EGTA (A) and verapamil (B) on CTC fluorescence induced by HWC-elicitor. The aged tuber discs were pre-treated with 1 mM CTC for 10 min, rinsed with distilled water, followed by either 1 mM EGTA, 100 μM verapamil or 10 mM Tris-HCl buffer (pH 7.4) for 10 min. Then either HWC-elicitor (100 μg/ml) or 10 mM Tris-HCl buffer was applied. Letters beside each line indicate the sequence of treatments.

Effects of HWC-elicitor, EGTA and verapamil on CTC-derived fluorescence

HWC-elicitor treatment reduced the CTC fluorescence level within 2 to 3 min of its application in a dose-dependent manner until at least 18 min after elicitation (Fig. 2). The buffer treatment as a control retained its CTC fluorescence almost at the same level during further incubation. In addition to this, EGTA and verapamil, the extracellular Ca2+ chelator and Ca2+ channel blocker, respectively, suppressed the elicitor-stimulated decrease in CTC-derived fluorescence (Fig. 3).

Luminol-mediated chemiluminescence in elicitor-treated tuber discs

Luminol-mediated chemiluminescence from elicitor-treated surfaces of discs was initiated around 8 min after treatment, and chemiluminescence per min gradually increased with further assay time (Fig. 4). This indicated that the oxidative burst in elicitor-treated tuber was induced several minutes after the elicitor-stimulated reduction of CTC fluorescence (Figs. 2 and 3). CTC fluorescence in isolated plasma membrane

To confirm whether CTC fluorescence is dependent on CaCl2 and CTC, we examined the relationship between CTC-fluorescence intensity and dosage of CaCl2 or CTC in Ca2+ free plasma membrane-rich fraction. The intensity of CTC fluorescence linearly increased depending on the concentrations of CaCl2 in the presence of sufficient fluorescence to a maximum equivalent to that of 2 mM CTC (Fig. 1). We used a 1 mM CTC solution in the following experiments because the concentration does not saturate the fluorescence intensity.
Fig. 4. Time-course changes in chemiluminescence from potato tuber discs treated with HWC-elicitor. Aged tuber discs were treated with luminol (2.5 mM) in 10 mM Tris-HCl buffer (pH 7.4) in the presence (■) or absence (○) of HWC-elicitor (1 mg/ml).

Fig. 5. Relationship between CTC-fluorescence intensity and dosage of CaCl₂ or CTC in plasma membrane-rich fraction. (A), Various concentrations of Ca²⁺ were added to the plasma membrane-rich fraction (0.5 mg protein/ml) in the presence of 10 μM CTC. (B), Various concentrations of CTC were added to the plasma membrane-rich fraction (0.5 mg protein/ml) in the presence of 1 μM CaCl₂.

CTC (10 μM; Fig. 5A). Conversely, the intensity also linearly increased depending on the concentrations of CTC in the presence of sufficient CaCl₂ (1 μM; Fig. 5B). The intensity of CTC fluorescence of the plasma membrane fraction reached a maximum at 15 μM CaCl₂ in the presence of a saturated concentration of CTC. Moreover, the pre-treatment of the membrane fraction with EGTA (1 mM) prevented CTC fluorescence, while post-treatment of the CTC-loaded membrane fraction with EGTA minimally influenced the fluorescence level (data not shown). These results indicated that the intensity of CTC fluorescence resulted from specific Ca²⁺-chelating by CTC in the presence of the plasma membrane.

Application of HWC-elicitor to the Ca²⁺-bound plasma membrane fraction immediately caused a decrease in CTC fluorescence 4 min after the elicitor application in a dose-dependent manner (Fig. 6). The fluorescence gradually decreased until at least 18 min after elicitation with wavy shifts of the fluorescence especially at high concentrations of HWC-elicitor (50 to 250 μg/ml).

Fig. 6. Time-course changes in CTC fluorescence in Ca²⁺-bound plasma membrane-rich fraction treated with HWC-elicitor at concentrations designated (μg/ml). The Ca²⁺-bound plasma membrane fraction (1 mg protein/ml) which was prepared as described in the materials and methods was loaded with 10 μM CTC and 1 μM CaCl₂.

DISCUSSION

Accumulating evidence suggests that there are marked differences in the responses of plant cell cultures and intact plant tissue⁷. Most data on Ca²⁺ in the signal transduction leading to plant defense responses have been obtained from cell cultures¹⁸. Our
study demonstrates Ca\(^{2+}\) movement on the plasma membrane in plant tissues treated with a pathogen-derived elicitor. In plants, two kinds of Ca\(^{2+}\) stores are believed to contribute to an increase in the concentration in the cells. One is an extracellular store in the cell wall, the other is an intracellular store such as the endoplasmic reticulum\(^{21}\). In pea tissues, an inhibitor of phospholipase C, which is responsible for the release of Ca\(^{2+}\) from intracellular stores, suppresses the accumulation of the phytoalexin induced by an elicitor\(^{28}\). Likewise, inhibitors of intracellular Ca\(^{2+}\) movement abolish the phytoalexin induction in oat leaves\(^{29}\).

In potato tubers, the elicitor-induced oxidative burst is inhibited by treatment with EGTA or verapamil\(^{29}\). Zook \textit{et al.} also showed that phytoalexin accumulation induced by arachidonic acid is prevented by EGTA\(^{38}\). We report here that the decrease in CTC-derived fluorescence and activation of the PAL gene induced by the HWC-elicitor were dramatically suppressed by EGTA or verapamil. EGTA is not thought to enter cells but to chelate apoplastic Ca\(^{2+}\). On the other hand, verapamil, a phenylalkylamine derivative, is an effective inhibitor of Ca\(^{2+}\) channels in a variety of membranes when applied to the intracellular surface of the channel, where it binds to the transmembrane pore\(^{30}\). Our pharmacological results suggest that the release of Ca\(^{2+}\) into cytoplasm from extracellular stores is involved in defense responses. However, we cannot rule out the possibility that intracellular stores also participate in the defense.

One objective of the study was to measure the timing of Ca\(^{2+}\) movement in relation to the elicitor-stimulated oxidative burst. In the present experiments, HWC-elicitor-stimulated reduction of CTC-fluorescence occurred much earlier than the oxidative burst. Therefore, HWC-elicitor may primarily cause the rapid release of Ca\(^{2+}\) into the cytoplasm of plant cells through the plasma membrane to activate the O\(_2\)-generating reaction. In plants, two kinds of enzymes are believed to contribute to O\(_2\)-generation in response to pathogen-derived elicitors; i.e. NADPH oxidase in the plasma membrane\(^{11,17,32}\) and peroxidase in the cell wall\(^{40}\).

Recently, a gene for the gp91\(^{phox}\) homolog that encodes a subunit of the neutrophil respiratory burst NADPH oxidase was cloned in rice\(^{19}\) and Arabidopsis\(^{17,32}\). An immunological study showed that calmodulin antagonists and EGTA inhibit elicitor-induced NADPH oxidase activity and the translocation of the cytosolic components of the oxidase such as p47\(^{phox}\) and p67\(^{phox}\) in tomato cells\(^{34}\). Moreover, because treatment with EGTA, verapamil and diphenyleneiodonium (an inhibitor of NADPH oxidase) inhibit the oxidative burst\(^{22,24}\), NADPH oxidase appears to be important in the oxidative burst in potato tubers.

Eighty percent of isolated plasma membrane vesicles were assumed to be sealed in the right-side-out state\(^{11}\). Ca\(^{2+}\) added to a suspension of the Ca\(^{2+}\)-free plasma membrane fraction may bind to the outside of the membrane vesicles. The HWC-elicitor-stimulated reduction of the CTC fluorescence may be a consequence of membrane perturbation in association with Ca\(^{2+}\) movement preceding the oxidative burst induced by the elicitor. However, we cannot address the regulatory mechanism of how the HWC-elicitor releases Ca\(^{2+}\) from the binding site of plasma membrane vesicles. Further experiments are needed to elucidate not only the relationship between the receptors for the fungal signals and release of Ca\(^{2+}\) from the membrane, but also the mechanism for activation of O\(_2\)-generating reaction associated with Ca\(^{2+}\). Furthermore, work is under way to evaluate the role of active oxygen species in signal transduction leading to activation of the defense genes.

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Literature cited


和 文 摘 要

三浦由雄・吉岡博文・朴 海準・川北一人・遠藤紀志：ジャガイモ疫病菌菌体エリシター処理を施したジャガイモ塊茎および原形質膜画分におけるカルシウムイオンの動向と活性酸素生成・防御遺伝子発現との関連

クロロテトラサイクリン（CTC）は生体膜等の疎水条件下にあるカルシウムイオンと結合して蛻光を発することが知られている。CTCで負荷したジャガイモ塊茎組織を疫病菌菌体エリシターで処理すると、処理濃度に依存して膜からカルシウムイオンの遊離を示唆する激発な蛻光の増減が観察された。また、この蛻光増減は、カルシウムチャンネルブロッカーであるペラバミルおよび細胞外カルシウムキレート剤であるEGTAの前処理で抑制された。カルシウムを用いて菌体エリシター誘導による活性酸素生成を経時的に測定したところ、蛻光の増減は活性酸素生成に先行することが明らかになった。さらに、原形質膜画分に負荷したCTCの蛻光強度は、菌体エリシター処理により速やかに減衰した。防御遺伝子であるフェニルアラニン・アンモニアリアーゼ（PAL）の発現に対するカルシウム薬害剤の影響を調べたところ、菌体エリシター誘導によるPAL mRNAの蓄積は、ペラバミルおよびEGTA処理により著しく抑制された。以上の結果は、菌体エリシター処理により速やかに遊離した宿主細胞の膜結合型カルシウムが活性酸素生成や防御遺伝子の発現において重要な役割を果たすことを見出される。