Measurement of Ca\(^{2+}\) Fluxes during Elicitation of the Oxidative Burst in Aequorin-transformed Tobacco Cells*

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We have employed suspension cultured aequorin-transformed tobacco cells to examine the involvement of Ca\(^{2+}\) in signal transduction of the oxidative burst. Use of cultured cells for this purpose was validated by demonstrating that the cells responded to cold shock quantitatively and qualitatively similarly to the intact transgenic plants from which they were derived. Stimulation of the oxidative burst in the cell suspension was achieved by administration of oligogalacturonic acid, Mas-7 (a peptide known to activate G proteins and Ca\(^{2+}\) fluxes), hypo-osmotic stress, or harpin (a protein from the pathogenic bacterium *Erwinia amylovora*). The latter failed to promote any detectable increase in cytoplasmic Ca\(^{2+}\) concentration, whereas each of the former three triggered a rapid rise in cytosolic Ca\(^{2+}\) followed by a return within seconds to basal Ca\(^{2+}\) levels. Peak Ca\(^{2+}\) concentrations induced by the former three elicitors were ~0.7, 1.4, and 1.3 \(\mu\)M, respectively. Three lines of evidence suggest that the observed Ca\(^{2+}\) pulses are essential to transduction of the oxidative burst signals by their respective elicitors: (i) inhibition of the Ca\(^{2+}\) transients with Ca\(^{2+}\) chelators or Ca\(^{2+}\) channel blockers prevented expression of the oxidative burst, (ii) introduction of exogenous Ca\(^{2+}\) into the same cells initiated the burst even in the absence of other inducers of the response, and (iii) the observed Ca\(^{2+}\) transients often returned to near basal levels well before any \(\text{H}_2\text{O}_2\) synthesis could be detected, suggesting that the Ca\(^{2+}\) influx is required to communicate the burst signal but not maintain the defense response. These data suggest that Ca\(^{2+}\) pulses serve frequently, but not invariably, to transduce an oxidative burst signal.

The oxidative burst constitutes one of the more rapid responses of a plant cell to pathogen attack (1). Within minutes of pathogen recognition, reactive oxygen species are generated that may promote cross-linking and lignification of the cell wall (2, 3), transcription of defense-related genes (4, 5), secondary metabolite biosynthesis (6), the hypersensitive response (4, 7, 8), and direct pathogen cytotoxicity (9, 10), depending on the plant species examined. In cultured cell systems, the oxidative burst can be promoted by isolated elicitors including harpin (11, 12), oligouronides (13), elicitors (14), purified fungal peptidases (15), and other molecules from the extracts of pathogens (7, 16). A biotic stimulus such as mechanical stress (17), the pesticide fenthion (18), cold shock (19), phosphatase inhibitors (4, 20), and hypo-osmotic stress (17) can also induce biosynthesis of reactive oxygen species in plant cells.

Because of its rapid expression, ease of assay, and similarity to the analogous defense response in human neutrophils (21), the oxidative burst has recently served as a model for exploring signal transduction pathways in plants. As the numbers of elicitors examined have risen, it has become increasingly clear that different elicitors may inaugurate independent signal transduction pathways that employ distinct sets of second messengers (1, 22). Indeed, the pathway initiated by oligouronides appears to require participation of phospholipase C but not phospholipase A (12, 23), whereas the pathway triggered by harpin from *Verticillium dahliae* requires exactly the converse (12). Although all of the independent signaling pathways are believed to converge with the assembly of an oxidase complex on the plasma membrane (24–26), it remains uncertain as to whether the various pathways also share a need for specific kinases or Ca\(^{2+}\).

Ca\(^{2+}\) is already believed to serve as a second messenger in such important plant processes as stomatal closure (27, 28), geotropism (29), control of circadian rhythms (30), pollen tube elongation (31, 32), regulation of plasmodesmata aperture (33), response to physical stress (34), and phytochrome phototransduction (35). Nevertheless, its accurate quantitation during cellular signaling events has until recently proven very difficult. Thus, \(^{45}\text{Ca}\)\(^{2+}\) binds avidly to cell walls, often masking the small changes in cytoplasmic \(^{45}\text{Ca}\)\(^{2+}\) that could indicate its function as a second messenger (36). Further, signal transduction pathways that might trigger release of \(^{45}\text{Ca}\)\(^{2+}\) from intracellular organelles are largely invisible to \(^{45}\text{Ca}\)\(^{2+}\)-based methodologies, because no net change in cellular \(^{45}\text{Ca}\)\(^{2+}\) content occurs. Although Ca\(^{2+}\)-sensitive fluorescent dyes have yielded useful information in a few plant systems (37), their general resistance to entry into plant cells has greatly limited their application (37). Indeed, most membrane-permeant esters of Ca\(^{2+}\)-dependent fluorophores hydrolyze to their impermeant forms in the cell wall, preventing their penetration to their intracellular destinations. Fortunately in 1991, Knight et al. (38) transformed tobacco plants with a Ca\(^{2+}\)-sensitive luminescent protein, termed aequorin, and demonstrated that resting levels of Ca\(^{2+}\) were insufficient to induce photon emission but that stimulus-induced Ca\(^{2+}\) increases generated readily measurable levels of bioluminescence. Since this initial discovery, aequorin-transformed plants have been exploited to quantitate intracellular Ca\(^{2+}\) fluxes accompanying such diverse processes/stimuli as direct mechanical perturbation (34, 38), gusts of wind (39), cold shock (38, 40), and circadian oscillations (30). Because the aequorin protein is expressed in the plant cell cytoplasm and because the intensity of light emitted by aequorin is directly proportional to Ca\(^{2+}\) concentration, measurement of aequorin-derived bioluminescence enables direct quantitation of Ca\(^{2+}\) transients in the transformed cytoplasm of the cell. In this report, we have employed suspension cultures of

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EXPERIMENTAL PROCEDURES

Materials—BAPTA-AM and ionomycin were obtained from Calbiochem (La Jolla, CA), whereas ruthenium red was purchased from Sigma. Coelenterazine was obtained from Molecular Probes (Eugene, OR). All other chemicals were reagent grade or of higher purity and were obtained from major chemical suppliers.

Elicitors—An OGA\textsuperscript{2} fraction that elicits the oxidative burst in all plant species tested to date was prepared as described previously (13). The OGA preparation used in these studies contained 0.5 ng/ml galac-turonide equivalents as determined by the method of Blumenkrantz and Asboe-Hansen (41). Harpin, a potent inducer of both the oxidative burst (11, 12) and the hypersensitive response (42), was a kind gift of Dr. Steven Bee (Cornell University). Mas-7, an active analog of mastoparan, and Mas-17, its inactive counterpart, were purchased from Calbiochem.

Aequorin-transformed Tobacco Suspension Cultures—Generation of aequorin-transformed tobacco (Nicotiana plumbaginifolia) plants has been previously described (38, 43). Transgenic seeds from an F\textsubscript{3} generation were a generous gift from Dr. Anthony J. Treweavas (University of Edinburgh). The seeds were sprouted on wet filter paper, and the seedlings in millimolar concentrations of either La\textsuperscript{3+} or EGTA (46), we next examined the sensitivity of our suspension cultures to the same reagents. As also shown in Fig. 1A, both La\textsuperscript{3+} and EGTA blocked most of the cold shock-induced increase in cytoplasmic Ca\textsuperscript{2+} concentration. Third, a crude proportionality was previously observed in the transgenic seedlings between the magnitude of the temperature decrease and the amplitude of the Ca\textsuperscript{2+} pulse. Using the transgenic cell cultures, we observed an analogous proportionality, wherein the intensity of the luminescence spike increased with the magnitude of the temperature drop (data not shown). Together these data argue that the transgenic tobacco cell suspension cultures constitute a reasonable model of the transgenic plant.

Determination of True Intracellular Ca\textsuperscript{2+} Concentration—Because the relationship between luminescence and Ca\textsuperscript{2+} concentration is double logarithmic, presenting data simply as a change in luminescence can greatly exaggerate the extent of any change in intracellular Ca\textsuperscript{2+} concentration. Therefore, we wrote a computer program to convert the observed luminescence signal directly into intracellular Ca\textsuperscript{2+} concentration using the equation described under “Experimental Procedures.” Fig. 1B presents the transformation of the data presented in Fig. 1A. This transformation reveals that in response to cold shock, the cytoplasmic Ca\textsuperscript{2+} concentration peaks at around 2.7 \muM, a value in the range observed for whole plants. Furthermore, addition of La\textsuperscript{3+} and EGTA reduced the peak Ca\textsuperscript{2+} level to less than 1 \muM, once again comparable with the results obtained by Knight et al. (46).

Some Elicitors of the Oxidative Burst Induce a Ca\textsuperscript{2+} Flux—With the ability to characterize the kinetics and magnitude of Ca\textsuperscript{2+} fluxes in cultured tobacco cells established, it was of interest to examine whether an intracellular Ca\textsuperscript{2+} pulse might participate in an elicitor-induced oxidative burst. We focused our investigation on the elicitors OGA, Mas-7, hypo-osmotic shock, and harpin primarily because of our previous experience with these elicitors. Specifically OGA, a component of the plant cell wall thought to be released during pathogen attack or

1 The abbreviation used is: OGA, oligogalacturonid acid.
The impact of elicitors on intracellular Ca$^{2+}$ levels was evaluated by mixing the elicitors with reconstituted aequorin cells and monitoring the increase in aequorin luminescence. As seen in Fig. 2A, OGA induces an influx of Ca$^{2+}$ into the cytoplasm that begins immediately following OGA addition and ends within 20 s of its initiation. The peak Ca$^{2+}$ concentration achieved in the experiment displayed here was 0.67 μM; however, this value varied from 0.28 to 0.9 μM, depending on the responsiveness of the particular flask of cells employed. Mas-7 also induces a rapid increase in cytoplasmic Ca$^{2+}$, although the magnitude (1.4 μM) and duration of the pulse are much larger than observed with OGA (Fig. 2). Mas-17, an inactive analog of Mas-7, has little effect on aequorin luminescence, confirming that the Mas-7 effect is indeed specific. Osmotic shock, unlike the other elicitors, triggers two contiguous phases of Ca$^{2+}$ release that last for a total of ~4–5 min (Fig. 2). The peak Ca$^{2+}$ concentration is $1.34 \pm 0.39 \mu M$ for the first pulse and $1.44 \pm 0.12 \mu M$ for the second ($n = 5$). Interestingly, harpin, even at concentrations sufficient to induce both the burst and the hypersensitive response does not cause an increase in aequorin luminescence, even when the luminescence measurements are extended to 40 min following stimulation. Because the signal to noise ratio in these studies is greater than 50:1 (actual data are shown without any noise reduction), it can be concluded that Ca$^{2+}$ is not necessary for transduction of the harpin-stimulated oxidative burst.

Using untreated cells from the same flasks, we simultaneously monitored the oxidative burst initiated by the above elicitors using a fluorimetric assay. Although the oxidative burst patterns for these elicitors have been previously published (12), they are presented here to allow a direct comparison of the kinetics of the Ca$^{2+}$ pulse with the kinetics of oxidant production by the same cells. Fig. 2B shows that the different elicitors, including harpin, induce bursts characterized by different lag periods following elicitor addition and by different rates of H$_2$O$_2$ production. Nevertheless, in all cases where a Ca$^{2+}$ transient is observed (Fig. 2A), the Ca$^{2+}$ influx precedes the biosynthesis of H$_2$O$_2$ (Fig. 2B). In fact, for each of the three Ca$^{2+}$-mediated pathways, the concentration of cytoplasmic Ca$^{2+}$ is seen to return to its unstimulated level before production of the oxidative species terminates. Importantly, this rapid expulsion of Ca$^{2+}$ from the cytoplasm cannot be an artifact of aequorin depletion, because cell permeabilization at the end of each experiment demonstrated that only a small fraction of the intracellular aequorin had been consumed (see “Experimental Procedures”). Further, the Ca$^{2+}$ influx cannot be dependent on oxidant production, because addition of sufficient catalase to eliminate any detectable H$_2$O$_2$ had no effect on the Ca$^{2+}$ influx.
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The traces presented here were chosen from several trials (nometry was conducted as described under “Experimental Procedures.”)

**The Observed Ca$^{2+}$ Pulses Participate in Transducing an Oxidative Burst Signal—**The observation that harpin can induce a burst without participation of Ca$^{2+}$ as a second messenger raises the question of whether the Ca$^{2+}$ influx stimulated by the other three elicitors is an epiphenomenon or an essential step in signaling their oxidative bursts. To investigate this issue, we decided to block the influx of Ca$^{2+}$ induced by OGA, Mas-7, and osmotic shock, and then monitor the effect on H$_2$O$_2$ biosynthesis. As shown in Fig. 3, A–F, incubation of cells with ruthenium red, a Ca$^{2+}$ channel inhibitor known to be active in plant cells (49), eliminates most of the elicitor-evoked Ca$^{2+}$ flux as well as most of the oxidative burst for all three elicitors. Furthermore, treatment of the cell suspensions with BAPTA-AM, a membrane-permeable intracellular Ca$^{2+}$ chelator, eliminated all oxidant production (data not shown). As expected, neither treatment with ruthenium red nor Ca$^{2+}$ chelator affected the harpin-induced burst, i.e. consistent with its inability to mobilize Ca$^{2+}$. These data suggest that Ca$^{2+}$ is indeed necessary for the Mas-7, OGA, and hypo-osmotic stress-stimulated oxidative bursts and that prevention of its influx is sufficient to block oxidant biosynthesis.

**Effect of Ca$^{2+}$ Influx on Burst Activity—**To learn whether Ca$^{2+}$ influx is itself sufficient for induction of the oxidative burst, we examined the effect of ionomycin, a Ca$^{2+}$ ionophore known to be active in both plants and animals, on generation of H$_2$O$_2$ by the coelenterazine-treated tobacco cells. As seen in Fig. 4, A and B, addition of 20 μM ionomycin to the aequorin-transformed cells induces both a rise in intracellular Ca$^{2+}$ levels (>4 μM) as well as a burst in H$_2$O$_2$ biosynthesis. In contrast, when EGTA is present in the culture medium, ionomycin-mediated induction of the oxidative burst is prevented (data not shown). These data suggest that Ca$^{2+}$ influx alone is sufficient to initiate a burst in responsive tobacco cells. However, in the course of these studies, we also observed that as the suspension cultures age (>36 h after transfer to fresh medium) and enter their elicitor-insensitive stage (13, 45), they also lose their ability to respond to ionomycin with oxidant biosynthesis (data not shown). That is, although ionomycin is still capable of inducing the anticipated Ca$^{2+}$ influx in these insensitive cells (as is also hypo-osmotic stress), the cells fail to respond with an oxidative burst. These data suggest that competency factors downstream of the Ca$^{2+}$ influx remain important in regulating the response of a plant to elicitation.

**DISCUSSION**

The advent of transgenic plant lines has significantly improved the technology for monitoring Ca$^{2+}$ fluxes in suspension cultured cells. Thus, in contrast to the more conventional use of 45Ca$^{2+}$ and/or Ca$^{2+}$-sensitive fluorophores, the aequorin methodology (i) requires no harsh methods for loading cells or preparing them for observation, (ii) is neither toxic to the plant cells nor to the investigator, (iii) allows for the collection of quantitative data at short (0.1 s) time intervals, thereby permitting accurate evaluation of the kinetics of a Ca$^{2+}$ transient, (iv) permits localization of the Ca$^{2+}$-sensitive protein in desired internal organelles by use of organelle targeting sequences, and (v) generates crude data with a signal to noise ratio generally exceeding 50:1, enabling characterization of processes involving even very small changes in cytoplasmic Ca$^{2+}$. With such desirable attributes, the aequorin technology should serve as the method of choice for investigating the involvement of Ca$^{2+}$ in many cellular signaling events.
Since the use of suspension cultured plant cells to investigate molecular aspects of plant behavior, the question has frequently arisen regarding the relevance of single cell studies to whole plant physiology. Unfortunately, responses to this concern have been largely inadequate, primarily because valid comparisons between whole plants and single cells/cell clusters have been difficult to design. Although much still remains to be resolved on this issue, the similar responses to cold shock of the aequorin transgenic plants and their derived suspension cultures suggests that single cells can in some cases yield data that very accurately reflect the behavior of the whole plants. Thus, Knight et al. (46) observed a Ca\textsuperscript{2+} transient in cold-shocked tobacco seedlings that was characterized by a 10-s width at half-height that rose abruptly to 2.3 \textmu M Ca\textsuperscript{2+} and fell slowly to its basal (nanomolar) level over a period of ~30 s. We also observed a cold shock-induced Ca\textsuperscript{2+} pulse in the transgenic suspension cultures with an ~8-s width at half-height that increased precipitously to 2.6 \textmu M Ca\textsuperscript{2+} and then declined more slowly to basal levels over a ~30 s total time period. Ca\textsuperscript{2+} fluxes in both the cell suspension cultures and whole seedlings were similarly inhibited by EGTA and La\textsuperscript{3+}, and in both systems the magnitudes of the cold shocks and Ca\textsuperscript{2+} transients were roughly proportional. Although physical barriers/inequities prevent similar comparisons of Ca\textsuperscript{2+} transients following mechanical perturbation, elicitor stimulation, or osmotic stress, the observation that these signals also trigger Ca\textsuperscript{2+} fluxes in both single cell and whole plant systems (38, 39, 50, 51) adds further weight to the contention that cultured cells can serve as useful models when properly validated. With this motivation in mind, it should be possible to explore in greater detail the role of Ca\textsuperscript{2+} pulses in signaling a variety of defense-related responses.
Although the studies presented here provide the first quantitative data on the kinetics and magnitudes of the Ca\(^{2+}\) transients induced by elicitors of a defense response, they represent by no means the first evidence for Ca\(^{2+}\) involvement in defense-related signaling. Indeed, several groups have reported that extracellular EGTA can inhibit the mobilization of selected disease resistance mechanisms (52, 53). Other workers have shown that introduction of exogenous Ca\(^{2+}\) into resting cells can initiate a defense response in the absence of the usually required elicitors (16, 54). Still other laboratories have demonstrated that Ca\(^{2+}\) channel blockers such as La\(^{3+}\) can prevent expression of a normal pathogen resistance mechanism when conditions are otherwise designed to stimulate its induction (52, 54). Nevertheless, it has never been determined wherein Ca\(^{2+}\) actually exerts control over processes leading to disease resistance. In the case of the oxidative burst, data presented here demonstrate that Ca\(^{2+}\) must enter the tobacco cell cytoplasm to initiate the burst, but the cation must not remain to sustain it. Thus, for the OGA-induced burst, cytoplasmic Ca\(^{2+}\) rises to \(\sim 0.7 \mu M\) and returns to basal levels within \(-20\) s (Fig. 2). H\(_2\)O\(_2\) biosynthesis, in contrast, is initially detected \(-2\) min following OGA addition, i.e. well after the Ca\(^{2+}\) concentration has returned to its resting level. Even with ionomycin-treated cells, where the primary stimulus for oxidant production is the ionophore-catalyzed entry of Ca\(^{2+}\) into the cell, the Ca\(^{2+}\) stimulus is seen to disappear long before the end product of the pathway (i.e. H\(_2\)O\(_2\)) is observed (Fig. 4). Clearly, these data indicate that Ca\(^{2+}\) serves only a transient role of communicating an upstream signal to a downstream effector. After performing this messenger function, Ca\(^{2+}\) appears to be no longer needed. Moreover, it can be argued that the proximal Ca\(^{2+}\)-dependent effector in the signaling pathway may be similarly transiently activated, returning to its resting state when the activating Ca\(^{2+}\) is removed. Signaling components such as the Ca\(^{2+}\)-dependent protein kinases (55, 56), calmodulin (57) and its responsive kinases (58, 59), Ca\(^{2+}\)-dependent phosphatases (60), Ca\(^{2+}\)-gated channels (61), and Ca\(^{2+}\)-activated phospholipases (62) are obvious candidates for this downstream effector.

As mentioned previously, elevation of elicitor concentration stimulates a roughly proportional increase in the amplitude of the Ca\(^{2+}\) transient and the magnitude of its downstream oxidative burst. In contrast, when the same comparison is conducted among unrelated elicitors of the oxidative burst, no such proportionality is observed. Indeed, Mas-7 was found to induce the largest Ca\(^{2+}\) influx yet stimulate the weakest oxidative burst. Because Ca\(^{2+}\) appears to be essential for transduction of the burst signals of at least three of the elicitors, it must be concluded that factors in addition to Ca\(^{2+}\) can significantly modulate the strength of a burst signal and that these unidentified factors are differentially activated by the various elicitors. Such regulatory characteristics require the involvement of bifurcating and reconverging signal transduction pathways.

During preparation of this manuscript, another article appeared describing the use of aequorin-expressing suspension cultured cells to evaluate Ca\(^{2+}\) transients during hypo-osmotic shock (51). Although their aequorin transgene was introduced directly into the cultured tobacco cells by infection with Agrobacterium tumefaciens, the response to low osmolarity was nevertheless very similar to that shown in Fig. 2, except the first Ca\(^{2+}\) peak was very small and no quantitative data on Ca\(^{2+}\) were presented. Because no evaluation of the consequent oxidative burst was conducted (51), further comparisons between the two studies are not possible.

Finally, it was admittedly surprising that harpin was found to trigger no Ca\(^{2+}\) influx during its stimulation of the oxidative burst (Fig. 2) and presumed induction of hypersensitive cell death (4). Obviously, its signaling pathway must be very different from those initiated by OGA, Mas-7, and hypo-osmotic stress. Previous work has also shown that harpin signaling is also distinct from that of a V. dahliae elicitor (12). Nevertheless, Ca\(^{2+}\)-independent transduction of an oxidative burst signal is not without precedence, because concanavalin A, in contrast to most other stimuli, induces human neutrophils to...
synthesize reactive oxidants in the absence of any change in cytoplasmic Ca\(^{2+}\) (63). Clearly much additional research will be required before the diversity of mechanisms that initiate and regulate the plant oxidative burst is fully understood.

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