Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 4120–4123, May 1995
Colloquium Paper

This paper was presented at a colloquium entitled "Self-Defense by Plants: Induction and Signalling Pathways," organized by Clarence A. Ryan, Christopher J. Lamb, André T. Jagendorf, and Pappachan E. Kolattukudy, held September 15–17, 1994, by the National Academy of Sciences, in Irvine, CA.

Role of phosphorylation in elicitation of the oxidative burst in cultured soybean cells

(oligogalacturonide/elicitor/hydrogen peroxide/plant defense/protein phosphorylation)

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ABSTRACT **The oxidative burst is likely the most rapid defense response mounted by a plant under pathogen attack, and the generated oxidant species may be essential to several subsequent defense responses. In our effort to characterize the signal-transduction pathways leading to rapid H_2O_2/O_2^- biosynthesis, we have examined the role of protein phosphorylation in this resistance mechanism. K-252a and staurosporine, two protein-kinase inhibitors, were found to block the oxidative burst in a concentration-dependent manner. When added during H_2O_2 generation, the burst was observed to rapidly terminate, suggesting that continuous phosphorylation was essential for its maintenance. Importantly, phosphatase inhibitors (calyculin A and okadaic acid) were found to induce the oxidative burst in the absence of any additional stimulus. This may suggest that certain kinases required for the burst are constitutively active and that stabilization of the phosphorylated forms of their substrates is all that is required for burst activity. In autoradiographs of elicited and unstimulated cells equilibrated with $^{32}PO_4^{3-}$, several phosphorylated polypeptide bands were revealed that could represent proteins essential for the burst.**

Protein phosphorylation has been demonstrated to regulate a myriad of processes in both the plant and the animal kingdoms. In the plant kingdom, protein phosphorylation appears to participate in hormone signaling (1–3), leaf and flower development (4), photosynthesis (5), blue-light responses (6), and even pollen self-incompatibility reactions (7). More recently, evidence for phosphorylation changes in plant defense signaling pathways has been mounting, suggesting that phosphatases and kinases may act as second messengers in disease-resistance mechanisms also. For example, Grosskopf *et al.* (8) have demonstrated that elicitors of phenylalanine ammonia-lyase and ethylene production elicit rapid changes in protein phosphorylation in tomato cells. Furthermore, they have shown that serine/threonine kinase inhibitors not only block elicitor-induced phosphorylation changes but also eliminate the induced plant defense responses (9). Second, Kauss and coworkers (10) found that protein-kinase inhibitors decrease elicitor-induced Ca^{2+} uptake and K^+ release but increase coumarin synthesis in parsley cells. Third, fungal elicitors derived from *Phytopthora megasperma* f. sp. *glycinea* trigger rapid changes in protein phosphorylation and induction of phytoalexins in parsley (11) and soybean cultures (12). Additionally, oligogalacturonides (degree of polymerization = 14–15) that induce proteinase inhibitors I and II have also been shown to elicit phosphorylation of a 34-kDa soybean plasma-membrane protein (13). Most significantly, a resistance gene (*Pto R*) that specifies resistance to *Pseudomonas syringae* pv. *tomato* has been cloned and shown to be a serine/threonine kinase with homology to other known plant and animal kinases (14).

A plant defense response that has recently been receiving considerable attention is the oxidative burst. The oxidative burst is one of the earliest known defense responses and results in the production of O_2^-/H_2O_2. The oxidative species produced are not only toxic to invading pathogens but also play important roles in many other physiological processes. Thus, reactive oxygen species have been shown to be necessary for the hypersensitive response (15), strengthening the cell wall (16), and transcription of genes (17). The oxidative burst, occurring within minutes of elicitor addition, also lends itself to dissection of its signal-transduction pathways because of its ease of assay and rapid termination. Earlier work using the purified oligogalacturonide elicitor oligogalacturonic acid (OGA; previously designated PGA; refs. 18–23) has provided evidence that signaling events preceding the burst include binding of OGA to a receptor (18), activation of G proteins (19), and stimulation of phospholipase C (20). Yet, many questions regarding the second messengers involved remain. In this paper, we address whether changes in protein phosphorylation are involved in regulation of the burst. We provide data to show that protein-kinase inhibitors inhibit the OGA-induced burst, while phosphatase inhibitors induce the burst in the absence of OGA. We also demonstrate that changes in protein phosphorylation occur during elicitation.

MATERIALS AND METHODS

Materials. Staurosporine and calyculin A were obtained from Sigma, while K-252a and okadaic acid were purchased from Calbiochem. All other chemicals were reagent grade or of higher purity and were obtained from major chemical suppliers.

Plant Cell Culture. Cell suspension cultures of soybean (*Glycine max* Merr. cv. Kent) were maintained in W-38 medium (24) as described (21). When an elicitation study was to be conducted, 9 ml of filtered cells were transferred to 100 ml of fresh medium and allowed to grow for approximately 36 h before use, unless stated otherwise.

Elicitors. An OGA fraction that elicits H_2O_2 production in soybean cell suspension cultures was purified, as described (21). The preparation used in this study contained 0.5 mg of galacturonic acid equivalents per ml, as determined by the method of Blumenkrantz and Asboe-Hansen (25).

Spectrofluorimeteric Determination of H_2O_2 Production. H_2O_2 production in cultured soybean cells was detected by

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Abbreviation: OGA, oligogalacturonic acid.
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monitoring the oxidative quenching of the fluorescent peroxidase substrate pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt; excitation at 405 nm, emission at 512 nm; Molecular Probes) as described (22, 23). Briefly, a 1.5-ml cell suspension was treated with 7 μl of a stock solution of 0.2 mg of pyranine per ml of water, transferred to a fluorimeter cuvette, and maintained in suspension in the spectrofluorimeter by mild stirring. OGA and any desired inhibitors were added at the start of stirring, and pyranine fluorescence was monitored continuously during the assay. All pharmacological agents were tested to ensure that they did not alter peroxidase activity (S. C. Dwyer, L. Legendre, P. F. Heinstein, P.S.L., and T. L. Leto, unpublished data).

Labeling of Phosphoproteins. A 3-ml soybean cell suspension was labeled for 15 min with 50 μCi of [^{32}P]NaH_2PO_4 (1 Ci = 37 GBq; ICN). The labeled suspension was then elicited with 30 μl of OGA (0.5 mg/ml stock), and cellular activity was stopped at the appropriate times with trichloroacetic acid (5% final concentration). The cells were then treated with protease inhibitors (a final concentration of 10 μg of leupeptin, 2 μg of pepstatin A, and 2 μg of α_2-macroglobulin per ml) and homogenized in a Dounce homogenizer, and the precipitate was washed four times with water-saturated ether. The radiolabeled proteins were then separated by SDS/PAGE and revealed by autoradiography.

RESULTS

Inhibition of the Oxidative Burst by K-252a. To investigate the role of phosphorylation in regulation of the oxidative burst, we tested the effect of two different serine/threonine kinase inhibitors: K-252a, a serine/threonine kinase inhibitor with broad specificity, was selected because it has been shown to inhibit kinases involved in activation of other plant defense responses, such as production of ethylene and activation of phenylalanine ammonia-lyase (8). Fig. 1 shows that K-252a inhibited the oxidative burst in a dose-dependent manner with an $IC_{50} \approx 0.15$ μM. Similar IC_{50} values have been obtained by Felix *et al.* (9) for inhibition of two defense responses in tomato cells. As can be seen in Fig. 1, K-252a can completely inhibit the burst at a concentration of 10 μM. Inhibition of the burst by K-252a did not involve an increase in the delay between elicitor addition and appearance of H_2O_2 or a decrease in the rate of oxidant production but a decrease in the total amount of H_2O_2 generated (data not shown). Furthermore, addition of inhibiting concentrations of K-252a during an ongoing burst terminated the burst (Fig. 2). These data suggest that continual protein phosphorylation is required for the oxidative burst.

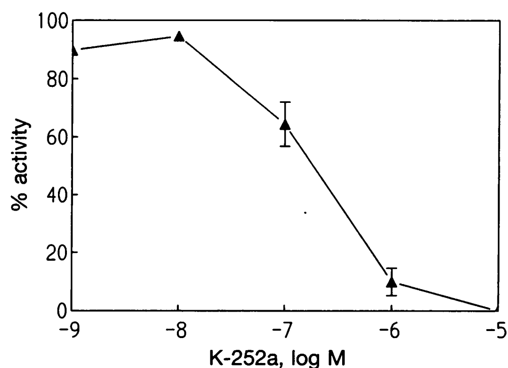

FIG. 1. Concentration dependence of the inhibition of the oxidative burst by the serine/threonine kinase inhibitor K-252a. Cells cultured for ≈36 h were treated with different concentrations of K-252a and 14 μl of OGA. The fluorescence transition due to H_2O_2-catalyzed oxidation of the fluorescent dye pyranine was monitored by using a Perkin–Elmer MPF44A spectrofluorimeter. The total amount of H_2O_2 produced was determined by measuring the vertical drop in fluorescence. Percent activity refers to the total amount of H_2O_2 generated and was calculated with respect to the transition in the absence of K-252a. Data presented here are the average of two independent experiments.

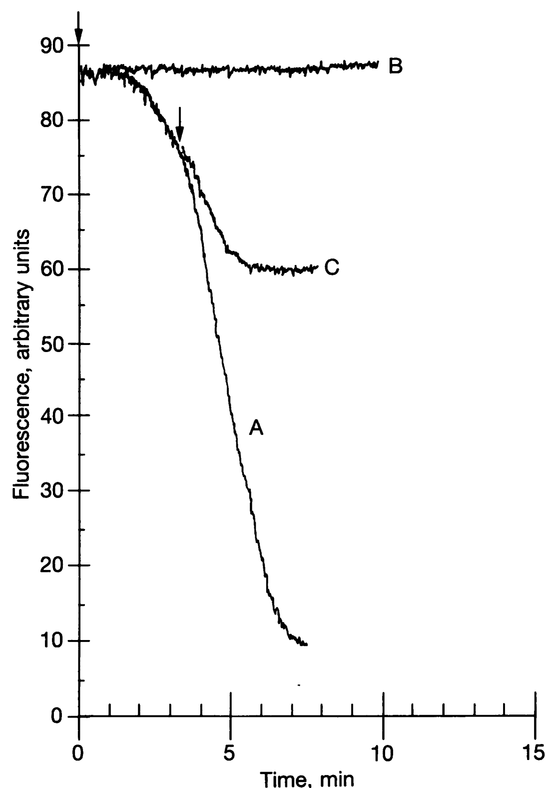

FIG. 2. Abrupt termination of the OGA-induced oxidative burst upon addition of the kinase inhibitor K-252a. Cells were treated with OGA alone (*A*), OGA and K-252a simultaneously (*B*), or OGA followed 3 min later by K-252a (*C*). The quenching of pyranine fluorescence due to H_2O_2 production was then continuously followed by spectrofluorimetry.

Inhibition of the Oxidative Burst by Staurosporine. To confirm the K-252a inhibition data, we examined the effect of staurosporine, another serine/threonine kinase inhibitor, on the same defense response. Staurosporine has been shown to inhibit mammalian kinases, as well as plant kinases (9). As shown in Fig. 3, staurosporine inhibits H_2O_2 production in a dose-dependent manner with an $IC_{50} \approx 0.9$ μM. As with K-252a, staurosporine could completely inhibit the burst. Similar results have been obtained by Schwacke and Hager

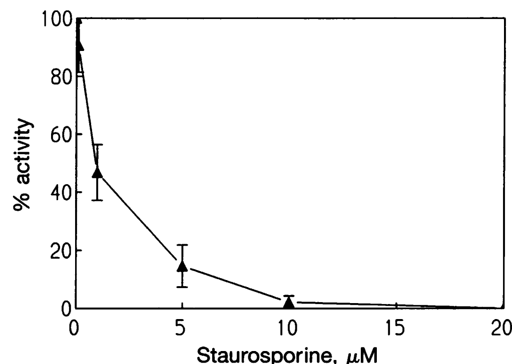

FIG. 3. Concentration dependence of the inhibition of the oxidative burst by the serine/threonine kinase inhibitor staurosporine. Assays were conducted essentially as described in the legend to Fig. 1, except that different concentrations of staurosporine were added. Data presented here are the average of two independent experiments.

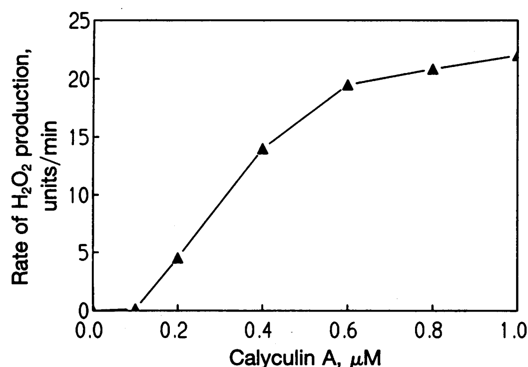

FIG. 4. Concentration dependence of the induction of the oxidative burst by the phosphatase inhibitor calyculin A. Cells cultured for ≈36 h were treated with various concentrations of calyculin A. The fluorescence transition due to H_2O_2-catalyzed oxidation of the fluorescent dye pyranine was monitored by using a Perkin–Elmer MPF44A spectrofluorimeter. The initial rate of decrease in pyranine fluorescence is plotted as a function of the concentration of calyculin A added. Similar curves were obtained in three independent experiments.

(26), who showed that staurosporine could inhibit elicitation of active oxygen species in spruce cells by crude fungal extracts. Together with the K-252a evidence, these data suggest that protein phosphorylation is important for elicitation and maintenance of the oxidative burst.

Elicitation of the Burst by Phosphatase Inhibitors. To verify that protein phosphorylation is important for activation of the burst and to determine whether phosphatase inhibitors can mimic the elicitor OGA, we evaluated the effect of two phosphatase inhibitors, calyculin A and okadaic acid. As seen in Figs. 4 and 5, both calyculin A and okadaic acid induced an oxidative burst in the absence of OGA. Though the rate of H_2O_2 production increased in a dose-dependent manner, the concentration for half-maximal stimulation (EC_{50}), as well as the maximal burst rate, varied considerably among cultures (EC_{50} from 0.18 to 0.3 μM for calyculin A and from 1.36 to 16 μM for okadaic acid). Yet, it is evident from these data that calyculin A is more effective than okadaic acid in eliciting the oxidative burst, in agreement with the ranking of potencies observed by Felix *et al.* (27) in monitoring other defense responses. Calyculin A and okadaic acid have frequently been used to distinguish between involvement of protein phosphatase 1 and 2, as calyculin A is more effective than okadaic acid in inhibiting protein phosphatase 1 (28). Our results may suggest that phosphatases involved in regulation of the H_2O_2 burst may be more closely related to protein phosphatase 1. Another interesting observation is that the burst induced by

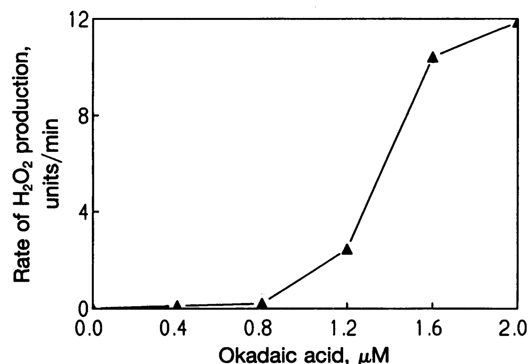

FIG. 5. Concentration dependence of the induction of oxidative burst by the phosphatase inhibitor okadaic acid. Assays were conducted essentially as described in the legend to Fig. 4, except that various concentrations of okadaic acid were added. Similar curves were observed in three independent experiments.

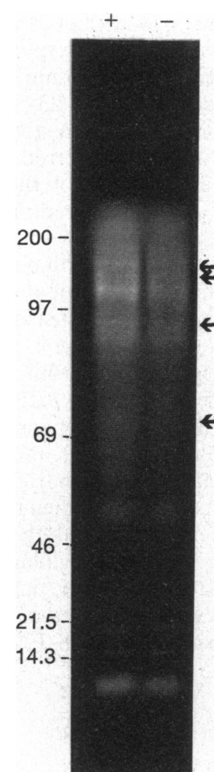

FIG. 6. Changes in protein phosphorylation during the OGA-induced oxidative burst. Cells cultured for ≈36 h and prelabeled with [^{32}P]NaH_2PO_4 were elicited with OGA (30 μl) for 5 min. H_2O_2 production was stopped with the addition of trichloroacetic acid to 5%. The acid precipitate was then washed with water-saturated ether. A total of 100 μg of protein was loaded in each lane and separated by SDS/PAGE. Phosphorylated proteins were revealed by autoradiography. −, Untreated cells; +, OGA-treated cells.

the phosphatase inhibitors continues for at least four times as long as the OGA-induced burst, with no end point detected after 1 h of continuous oxidase activity. This implies that protein dephosphorylation is probably critical in downregulation of the oxidative burst.

Changes in Protein Phosphorylation Upon Elicitation by OGA. Labeling proteins with $^{32}PO_4^{3-}$ revealed that several changes in protein phosphorylation occur during elicitation. Upon OGA addition, 65-, 115-, 150-, and 160-kDa polypeptides appear to be phosphorylated (Fig. 6). These polypeptide bands could represent proteins crucial for the oxidative burst.

DISCUSSION

In this paper we have provided evidence for the involvement of protein phosphorylation in the rapid oxidative burst. We have shown that the protein kinase inhibitors K-252a and staurosporine inhibit the burst at IC_{50} values comparable to those obtained for other plant defense responses (9). We have also shown that phosphatase inhibitors can induce a burst in the absence of an elicitor. Since addition of phosphatase inhibitors should lead to elevation of the level of the phosphate content of constitutively phosphorylated proteins, it would appear that an increase in phosphorylation of some proteins may be sufficient to induce the burst and that the kinases involved in the burst are constitutively active. Furthermore, on the basis of the requirement of Ca^{2+} for the oxidative burst (26), it is conceivable that some of the kinases involved are Ca^{2+}-dependent kinases. Some of the proteins identified in the autoradiograph could be those essential to the oxidative burst.

While characterization of the elicitor-induced phosphorylation events in cultured soybean cells is admittedly scanty, a few

comparisons with the roles of kinases and phosphatases in the neutrophil oxidative burst can still be drawn. As in soybeans, the neutrophil burst is inhibited by serine/threonine kinase inhibitors (29). However, in the neutrophil system at least one signaling pathway involves the activation and migration of protein kinase C to the membrane prior to start of the burst (29). While Ca^{2+}-dependent protein kinases are present in plant cells (30), if their participation in the oxidative burst is required, they must be constitutively active, since our data shows that simple inactivation of phosphatases can independently elicit the burst. Such induction of the burst by phosphatase inhibitors is not seen in neutrophils (31).

As noted earlier, the plant oxidative burst has been shown to be under control of G proteins and phospholipase C. In this paper we have demonstrated that changes in protein phosphorylation can also control the burst. Questions regarding the chronological order of these signaling events still remain to be addressed. Nevertheless, identification of several transduction intermediates has now allowed us to obtain handles on the signaling cascade that should permit elucidation of the second messenger chronology by the judicious use of inhibitors.

This work was supported by National Science Foundation Grant MCB 9303929.

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