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The protein phosphatase inhibitor calyculin A mimics elicitor action in plant cells and induces rapid hyperphosphorylation of specific proteins as revealed by pulse labeling with [^{33}P]phosphate

(tomato cells/alkalinization/ethylene/signal transduction/protein phosphorylation)

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ABSTRACT **Suspension-cultured tomato cells react to microbial signals, so-called elicitors, with rapid alkalinization of the growth medium and increased biosynthesis of the stress hormone ethylene. These responses to elicitors can be blocked by staurosporine and K-252a, two specific inhibitors of protein kinases. Here we show that calyculin A, a potent inhibitor of protein phosphatases, mimics the action of elicitors and, at nanomolar concentrations, induces medium alkalinization as well as a strong increase in the activity of 1-aminocyclopropane-1-carboxylate synthase, the key enzyme of ethylene biosynthesis. Both responses were strongly inhibited by K-252a, and calyculin A induced both responses more rapidly than did a fungal elicitor, xylanase. For example, the lag phase for medium alkalinization was only 0.2–0.4 min for calyculin A, compared with 2 min for xylanase. To study changes in the dynamics of protein phosphorylation, cells were labeled with 30-sec pulses of [^{33}P]orthophosphate. Calyculin A strongly increased phosphorylation of several polypeptide bands within 40 sec of treatment. The same phosphorylated bands also appeared in response to xylanase, but only after a lag phase of 2–3 min. These results show that the protein phosphatase inhibitor calyculin A leads to rapid hyperphosphorylation of specific proteins in cultured cells and indicate that elicitor action could be based on inhibition of a protein phosphatase as well as on activation of a protein kinase.**

Perception of signal molecules plays a key role in the interaction of plants with symbiotic (1) and pathogenic (2, 3) microorganisms. Signal molecules derived from pathogens, so-called elicitors, induce an array of biochemical responses collectively known as the defense response (2, 3). They also cause rapid, transient increases in the phosphorylation status of specific proteins (4–6), and these changes in phosphorylation as well as the early biochemical responses to elicitors can be blocked by inhibitors of protein kinases, such as staurosporine and K-252a (5–9). This has been taken as an indication that chemosensory transduction of the elicitor signal depends on phosphorylation of specific proteins, as has been shown for chemoperception by bacteria (10) as well as by eukaryotic microorganisms (11) and animals (12). In plants, evidence for a participation of protein phosphorylation in signal transduction has also been provided for light signals (13), for oligogalacturonide signals related to the wound response (14), and for hormone stimuli such as cytokinins, auxins, and ethylene (15, 16).

Models for chemosensory transduction are generally based on the notion that binding of a signal molecule to its receptor activates a protein kinase, resulting in phosphorylation of proteins involved in signal transduction (10–12). However, since protein phosphorylation is transient and reversible, an increased phosphorylation status of such signal-transducing proteins might equally well be brought about by inhibition of the corresponding protein phosphatases. Indeed, regulation of metabolic processes in animal cells involves activation and inactivation both of protein phosphatases and of protein kinases (17). Protein phosphatases 1 and 2A have been described in plants (18), and they too have been implicated in the regulation of various metabolic enzymes (19–22).

Here, we use calyculin A and okadaic acid, two potent inhibitors of protein phosphatases 1 and 2A (23), to study the potential involvement of protein dephosphorylation in chemosensory transduction in plant cells. We employ fungal xylanase as a model elicitor, a protein known to induce a range of defense responses in tobacco (16, 24, 25) and tomato (6, 7). We examine two typical early responses of suspension-cultured tomato cells to elicitors—namely, the rapid increase in pH of the growth medium (5, 6) and the increase in activity of the key enzyme of ethylene biosynthesis (26), 1-aminocyclopropane-1-carboxylate (ACC) synthase. We show that calyculin A induces medium alkalinization and ACC synthase activity more rapidly than the fungal elicitor itself. Using pulse labeling of cells with [^{33}P]phosphate, we further demonstrate that calyculin A causes hyperphosphorylation of specific polypeptide bands within 40 sec, whereas xylanase promotes phosphorylation of the same polypeptide bands after 2–3 min. These results lead to the conclusion that the proteins important for signal transduction are continually phosphorylated and dephosphorylated even in the absence of a stimulus and that inhibition of a protein phosphatase may be as significant for induction of elicitor responses as activation of a protein kinase.

MATERIALS AND METHODS

Plant Materials, Elicitors, and Inhibitors of Protein Kinases and Protein Phosphatases. The tomato cell suspension line Msk8 was maintained and subcultured as described (27). Cells were used for experiments 6–10 days after subculture. A commercial preparation of xylanase from *Trichoderma viride* (Fluka) was used as elicitor. Stock solutions (2 mM) of the protein kinase inhibitor K-252a (Fluka) or the protein phosphatase inhibitors calyculin A and okadaic acid (LC Services, Woburn, MA) were prepared in dimethyl sulfoxide.

Extracellular Alkalinization. The extracellular pH was monitored with a combined glass electrode in 2-ml aliquots of the suspension incubated in open flasks on a shaker (5). Cell suspensions were treated with xylanase (10 μg/ml), calyculin A (500 nM), K-252a (500 nM), or equivalent volumes of the solvent (dimethyl sulfoxide or water) as indicated.

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Abbreviation: ACC, 1-aminocyclopropane-1-carboxylic acid.

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Assays of ACC Synthase, ACC Oxidase, and Phenylalanine Ammonia-Lyase. ACC synthase activity was assayed in permeabilized cells (28). ACC oxidase activity was determined by measuring the rate of ethylene production in cells supplemented with a saturating concentration of ACC (27). In brief, cells (≈0.2 g of fresh weight in 1 ml of medium) were placed in 6-ml glass tubes and incubated on a rotary shaker at room temperature. After the addition of 0.1 mM ACC, tubes were closed with rubber caps, and ethylene accumulated in the gas phase was assayed 30 min later by gas chromatography (28). Phenylalanine ammonia-lyase activity was measured in tissue homogenates (7).

Assay of Protein Phosphatase. A batch of cells was homogenized in 50 mM Tris·HCl, pH 7.5/15 mM 2-mercaptoethanol/0.1 mM EDTA/0.1% bovine serum albumin. The crude homogenate was tested with the protein phosphatase assay system according to the protocol of the manufacturer (GIBCO/BRL). This assay employs ^{32}P-labeled phosphorylated phosphorylase A as a substrate, a standard substrate for protein phosphatases 1 and 2A (18).

Pulse Labeling of Phosphoproteins with [^{33}P]Phosphate. Aliquots (0.2 ml) of cell suspension (≈70 mg of cells) were added to carrier-free [^{33}P]phosphate (10 μCi = 370 kBq). Labeling was stopped after 30 sec by adding 0.3 ml of 10% (wt/vol) trichloroacetic acid/10 mM ATP and freezing in liquid nitrogen. Samples were thawed in a sonicator bath, and pellets were washed three times with 4:1 (vol/vol) acetone/100 mM Tris·HCl (pH 8.0) by resuspension and centrifugation. Pellets were extracted with 100 μl of SDS sample buffer (29) at 95°C for 5 min. After centrifugation, 25-μl samples of the supernatants were subjected to SDS/PAGE (29) and autoradiography.

RESULTS

The tomato cells used in this study have been found to react to fungal xylanase (6) and other elicitors (5, 6) with rapid alkalinization of their culture medium. In accordance with these previous results, xylanase was found to induce alkalinization after a lag phase of about 2 min (Fig. 1*A*). This response was completely blocked in cells pretreated with the protein kinase inhibitor K-252a (Fig. 1*B*). When given in midcourse of the response, K-252a stopped further alkalinization within ≈40 sec and then caused a rapid drop in extracellular pH (Fig. 1*A*), suggesting that maintenance of the elevated pH depended on *de novo* protein phosphorylation and that the rapid decay of the induced state might be caused by protein phosphatase action.

To examine the possible role of protein phosphatases, we tested calyculin A and okadaic acid, two potent inhibitors of protein phosphatases 1 and 2A (17, 23, 31), for their effects on the alkalinization response. When calyculin A (500 nM) was added to the cells, it induced a pH increase similar to that provoked by xylanase, after a lag of only 20–40 sec (Fig. 1*A*). This alkalinization was inhibited strongly although not completely when K-252a was added before calyculin A (Fig. 1*B*). K-252a could still partially reverse the alkalinization response when given within 2 min after calyculin A, but it no longer affected the pH response when given 15 min after calyculin A (Fig. 1*A*). Calyculin A induced significant medium alkalinization down to concentrations of 10 nM (Fig. 2*A*). In contrast, okadaic acid caused only slow and weak extracellular alkalinization even at concentrations up to 1 μM (Fig. 2*B*).

When the two compounds were tested for inhibition of protein phosphatase activity in homogenates of the tomato cells (Fig. 3), both displayed dose–response curves expected from work with other tissues (17, 18). Okadaic acid yielded a typical two-step curve with IC_{50} values of ≈100 pM and ≈100 nM for the two steps of inhibition (17, 18, 31). Calyculin A was active at even lower doses and, although the first step of inhibition was less clear and might actually comprise more

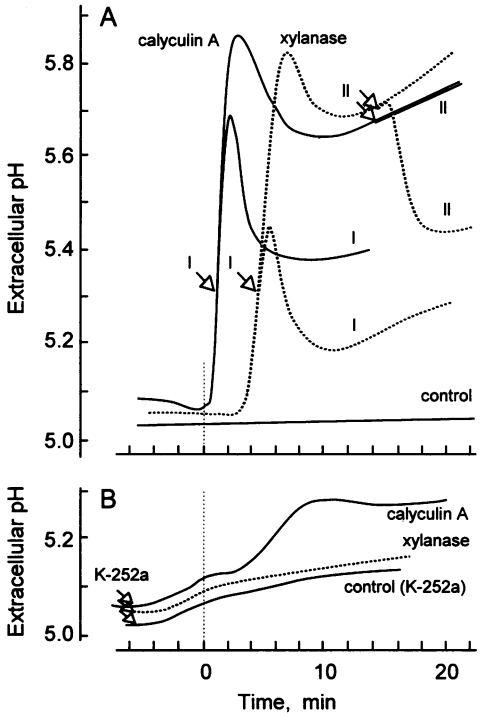

FIG. 1. Alkalinization of the growth medium by tomato cells in response to xylanase, calyculin A, and K-252a. (*A*) Tomato cells were treated with either xylanase (10 μg/ml) or calyculin A (500 nM) at time zero and with K-252a (500 nM) as indicated by the arrows. (*B*) Cells pretreated for ≈5 min with K-252a (500 nM) were treated with either xylanase (10 μg/ml) or calyculin A (500 nM) at time zero. Control cells received K-252a only.

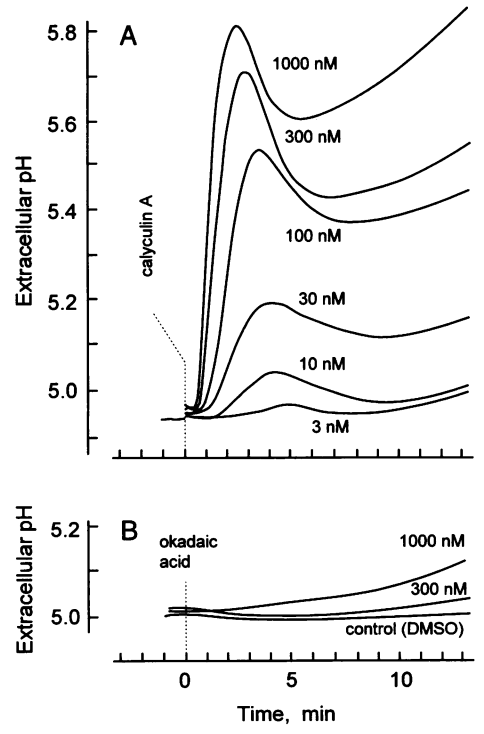

FIG. 2. Effect of various doses of calyculin A (*A*) and okadaic acid (*B*) on the extracellular pH of tomato cells. DMSO, dimethyl sulfoxide.

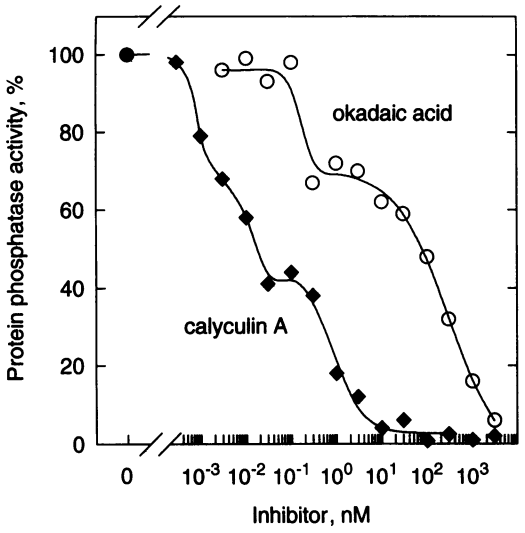

FIG. 3. Dose–response curve for the inhibition of protein phosphatase in tomato cell homogenates by calyculin A (◆) and okadaic acid (○). ^{32}P-labeled phosphorylated phosphorylase A was used as substrate and maximal activity (100%) was 4.6 nmol of phosphate released per minute per gram of cells in the experiment with calyculin A and 7.4 nmol per minute per gram of cells in the experiment with okadaic acid, respectively.

than one step, IC_{50} values of ≈3 pM and ≈1 nM were observed.

Induction of ethylene biosynthesis is another early response of plant cells to elicitors (reviewed in ref. 30). In suspension-cultured tomato cells, the first sign of this response is an induction of ACC synthase activity, apparently at the posttranscriptional level (27). Xylanase rapidly and strongly induced ACC synthase (Fig. 4). Compared with the low basal level in control cells, ACC synthase activity increased 50- to 100-fold within 60 min of stimulation. The lag phase of induction varied between 5 and 10 min for different batches of cells and was 10 min in the experiment shown in Fig. 4. Calyculin A provoked a similarly strong increase in

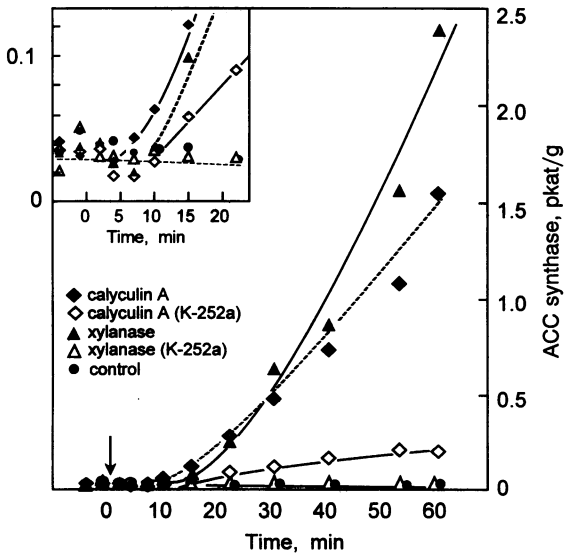

FIG. 4. Effect of xylanase, calyculin A, and K-252a on the activity of ACC synthase in tomato cells. Cells pretreated for 5 min with K-252a (500 nM, open symbols) or an equivalent volume of the solvent, dimethyl sulfoxide (filled symbols), were treated with either xylanase (10 μg/ml) or calyculin A (500 nM) at time zero. Control cells received dimethyl sulfoxide only. (*Inset*) Values for early time points on an expanded scale.

ACC synthase activity (Fig. 4). The lag phase for induction was 8 min in the experiment shown in Fig. 4 (*Inset*) and, within the resolution of the assay, consistently appeared to be about 2 min shorter than that observed for xylanase. Pretreatment of cells with K-252a completely inhibited induction of ACC synthase by xylanase but reduced stimulation by calyculin A only partially (Fig. 4).

A dose–response curve was established for the induction of ACC synthase by calyculin A. Half-maximal stimulation was observed at ≈100 nM, and the maximum level of increase was maintained for concentrations between 200 nM and 5 μM (Fig. 5*A*). In cells treated with xylanase, low doses of calyculin A, up to 60 nM, showed an additive effect, but at higher concentrations, induction was at the same level as with xylanase alone. Dose–response curves were also established for the effects of calyculin A on the second enzyme in ethylene biosynthesis, ACC oxidase (Fig. 5*B*), and on phenylalanine ammonia-lyase, two enzymes typically induced by elicitors in the tomato cells as well (27). Compared with ACC synthase, induction of these enzymes, thought to occur primarily at the transcriptional level (2, 27), was slower: clearly elevated levels could be measured only after a treatment of several hours (27). Calyculin A also induced ACC oxidase and phenylalanine ammonia-lyase, but only at concentrations between 20 and 100 nM (Fig. 5 *B* and *C*). Induction of ACC oxidase and phenylalanine ammonia-lyase by xylanase was strongly inhibited by concentrations of

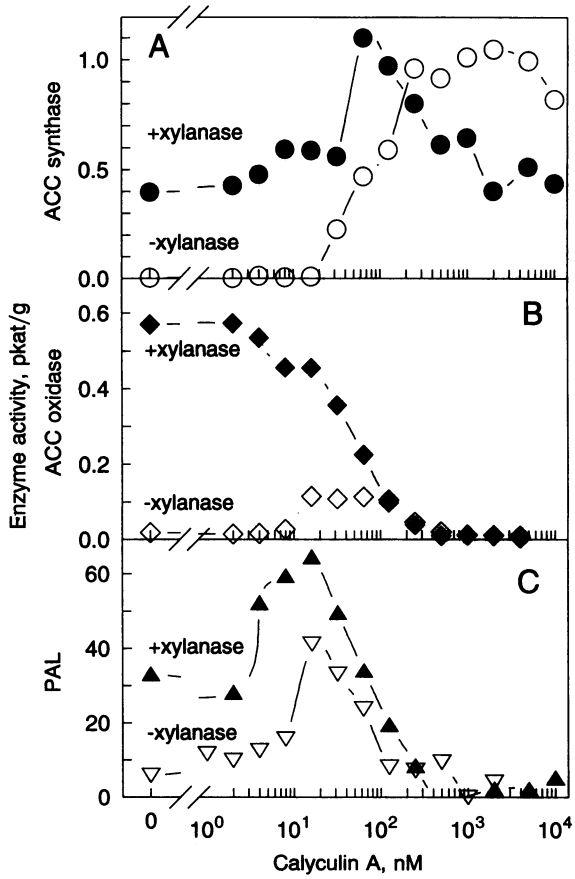

FIG. 5. Dose–response curves for the effect of calyculin A on ACC synthase (*A*), ACC oxidase (*B*), and phenylalanine ammonia-lyase (PAL) (*C*). Cells were treated with various doses of calyculin A either alone (open symbols) or together with xylanase (10 μg/ml) (filled symbols). (*A*) ACC synthase was measured after a treatment of 1 hr. (*B*) ACC oxidase was determined in the interval between 4.0 and 4.5 hr of incubation. (*C*) PAL was measured after 4 hr of incubation.

calyculin A above 50–100 nM (Fig. 5 *B* and *C*), indicating that the inhibitor disturbs the induction process in the long run.

To study the effect of xylanase and calyculin A on the dynamics of protein phosphorylation, we pulse-labeled the cells with [^{33}P]orthophosphate for 30 sec at various times after addition of the stimuli (Fig. 6*A*). Pulse-labeling has been shown to be suitable to label phosphoproteins with a high turnover of their phosphate groups (5, 6), and the use of ^{33}P instead of ^{32}P as a label greatly increased resolution on autoradiograms. To minimize indirect effects of the extracellular pH on phosphate uptake, cells were buffered with 10 mM Mes/KOH at pH 5.1. Under these conditions, the alkalinization response was still measurable and occurred with the same kinetics as in unbuffered cells but led to a pH increase of only 0.1 unit during the experiment (Fig. 6*A*). A series of control experiments showed that uptake of the radioactive phosphate occurred at the same rapid rate throughout the experiment in both the presence and the absence of stimuli: >90% of the label was taken up by the cells, and 0.5–1% of the label was incorporated into the trichloroacetic acid-insoluble fraction within the 30 sec of the pulse. SDS/PAGE of this fraction, followed by autoradiography, revealed a large number of bands in untreated control cells (first lane in Fig. 6 *B* and *C*), indicating rapid phosphorylation of many cellular proteins. When calyculin A was added to the cells, the first sample, taken after 20 sec (lane 2 in Fig. 6*B*; labeling had started 10 sec before addition of calyculin A) showed the same pattern of phosphoprotein labeling as control cells. Subsequently, several newly labeled bands appeared, most prominently among them four bands of 51, 27, 26, and 20.5 kDa (arrows in Fig. 6*B*). In total, >10 new bands could be observed consistently on one-dimensional SDS/PAGE, and these new bands became first visible at the onset of the alkalinization response. In addition, a somewhat more intense overall labeling was observed after 1–1.3 min. When xylanase was added to the cells, the pattern of labeled phosphoproteins was indistinguishable from controls for the first 2 min, but then, at the beginning of the alkalinization, showed the same marked changes as the ones apparent in cells treated with calyculin A (Fig. 6*C*). In particular, the newly phosphorylated bands marked with arrows were in the same position and displayed a relative intensity similar to that of the corresponding bands from the calyculin A treatment.

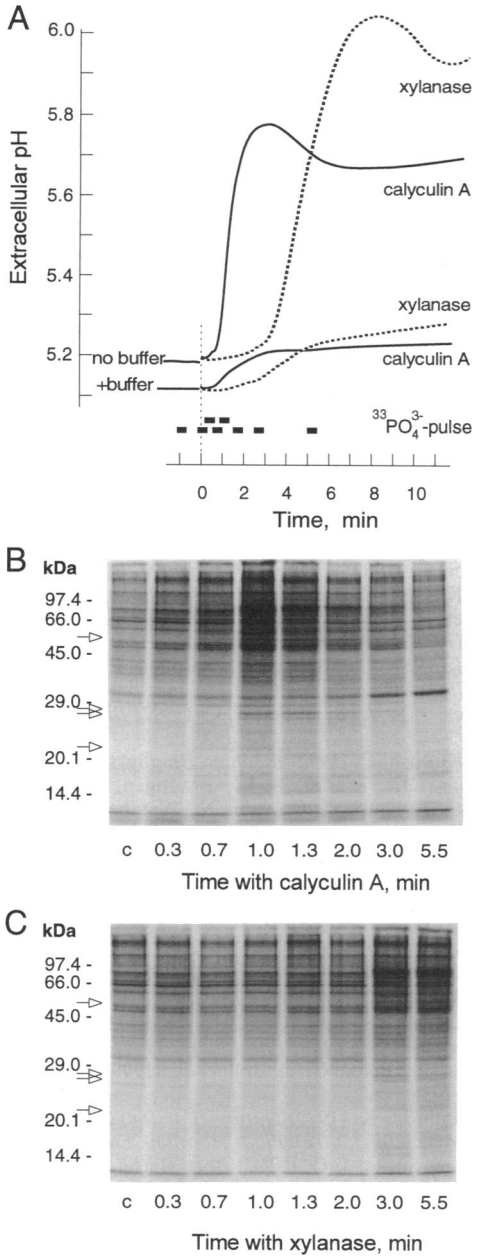

FIG. 6. Time course of alkalinization and of changes in protein phosphorylation in response to xylanase and calyculin A. (*A*) Effect of xylanase (10 μg/ml) or calyculin A (500 nM) on the extracellular pH in the presence or absence of 10 mM Mes/KOH buffer at pH 5.1. Bars above the time scale indicate the intervals used in *B* and *C* for the [^{33}P]phosphate pulses. (*B* and *C*) SDS/PAGE and autoradiography of cell extracts from tomato cells treated with calyculin A (*B*) or xylanase (*C*) in the presence of 10 mM Mes/KOH buffer at pH 5.1 and labeled with 30-sec pulses of [^{33}P]phosphate. First lane (c), extract of cells pulse-labeled 1 min before the remaining cells received xylanase or calyculin A.

DISCUSSION

We have previously shown that inhibitors of protein kinases such as K-252a rapidly block elicitor responses, even when applied in midcourse (ref. 5; see also Fig. 1) and that radioactivity disappears rapidly from the newly labeled phosphoproteins in the presence of K-252a (5). These findings indicate that protein kinase activity is important not only to initiate elicitor response but also to maintain it and that the rapid reversion of the elicitor effects by K-252a may be connected to protein phosphatase action.

We used calyculin A and okadaic acid, two inhibitors of protein phosphatases 1 and 2A (17, 23, 31), to study this possibility. While calyculin A has barely been used yet in plants (32), okadaic acid has been frequently employed as a tool to study regulation of metabolic enzymes (19–22) and of signal transduction processes (15, 16). In particular, okadaic acid has recently been reported to induce accumulation of pathogenesis-related proteins in tobacco in a similar way as the elicitor xylanase and the stress hormone ethylene (16). In our study, however, okadaic acid proved to be unsuitable as a probe for the early events in signaling, since at concentrations up to 1 μM it caused only slow and weak extracellular alkalinization (Fig. 1*B*) and was therefore not further studied. Calyculin A, in contrast, mimicked this early elicitor action and induced significant medium alkalinization down to concentrations of 10 nM (Fig. 2*A*). The simplest explanation for the differential effect of the two compounds is their differential affinity for different protein phosphatases, calyculin A showing much higher affinity than okadaic acid for protein phosphatase 1 (23). However, concentrations of calyculin A with maximal effect in cells (>100 nM) completely inhibit both classes of phosphatases in cell-free preparations, and differences in cell permeation or inactivation of the inhibitors may also play a role, making it difficult to pinpoint the type of protein phosphatase involved.

Calyculin A induced medium alkalinization and ACC synthase activity faster than did the elicitor xylanase (Figs. 1 and 2), a finding consistent with the idea that it acts downstream in the signal transduction pathway. Whereas medium alkalinization might be regulated directly by the phosphorylation of an unidentified ion channel or pump, induction of ACC synthase appears to be more indirect since it requires protein synthesis (27) and since ACC synthase activity is resistant to treatment with protein phosphatases *in vitro* (P.S., G.F., and T.B., unpublished work). Calyculin A also induced ACC oxidase and phenylalanine ammonia-lyase activities, although induction of these two enzymes, thought to be regulated at the transcriptional level (2, 27), occurred with a more complex dose dependency (Fig. 5 *B* and *C*). Stimulation was observed at low concentrations (20–100 nM), but concentrations inducing ACC synthase and alkalinization maximally were inhibitory and inhibited also the stimulation of these two enzymes by elicitor. Induction of these slower responses was measured after 4–4.5 hr of treatment, and prolonged inhibition of protein phosphatases or unknown side effects of calyculin A might be toxic for cellular processes such as transcription.

Rather than study the long-term effects of calyculin A, we tried to show its action on protein phosphorylation within the first few minutes of application, the time span necessary to establish the alkalinization response. Pulse-labeling with radioactive orthophosphate has been shown to be the method of choice to label phosphoproteins with a high turnover of their phosphate groups (5, 6). For this study we reduced the time of labeling to 30 sec and used ^{33}P instead of ^{32}P as a label. In addition to lowering the biohazard, the lower energy of the β radiation of ^{33}P greatly increased resolution on autoradiograms. Changes in the pattern of labeled phosphoproteins first became visible in cells labeled between 10 and 40 sec of treatment with calyculin A (Fig. 6*B*). The same set of changes was also observed in cells treated with the elicitor xylanase, although with a longer lag phase (Fig. 6*C*). These changes coincided in both cases with the onset of the alkalinization response. More than 10 new bands could be observed consistently after one-dimensional SDS/PAGE, a clear advantage of the superior resolution of the autoradiograms provided by labeling with ^{33}P as compared with ^{32}P.

Calyculin A was found to mimic elicitor action in several aspects, suggesting that inhibition of protein phosphatases by calyculin A is sufficient to induce elicitor responses, as long as the K-252a-sensitive protein kinase(s) is active. This is compatible with the hypothesis that the proteins critical for signaling are continually phosphorylated and dephosphorylated even in the absence of stimuli and that the phosphorylation status of the relevant proteins is low in the basal state because the protein phosphatases involved are more active than the corresponding protein kinases. In this highly dynamic system, inhibition of protein phosphatases by calyculin A may be sufficient to cause hyperphosphorylation of the relevant phosphoproteins within a few seconds and thereby to induce medium alkalinization and ACC synthase activity.

In conclusion, our data indicate that chemosensory transduction of elicitor signals in plant cells is based on a finely balanced system of protein phosphorylation and dephosphorylation. It will be interesting to find out whether chemosensory transduction in plants is primarily connected with a reduced activity of a protein phosphatase, with an increased activity of a protein kinase, or with a combination of both.

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