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# The protein kinase inhibitor, K-252a, decreases elicitor-induced $Ca^{2+}$ uptake and $K^+$ release, and increases coumarin synthesis in parsley cells

Uwe Conrath, Wolfgang Jeblick and Heinrich Kauss

*FB Biologie, Universität Kaiserslautern, Postfach 3049, D-6750 Kaiserslautern, Germany*

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**An elicitor preparation from fungal cell walls known to induce coumarin synthesis in suspension-cultured parsley cells also elicits a rapid and transient $Ca^{2+}$ uptake, $K^+$ release and external alkalinization, and increases uptake of $^{45}Ca^{2+}$ into the cells. The latter three responses were inhibited by the protein kinase inhibitor K-252a at 0.2 μM. Elicitor-induced coumarin synthesis, a process which requires gene activation, was greatly enhanced by K-252a. These results suggest that protein phosphorylation might be involved in the initial steps of signal transduction as well as in the long-term induction of coumarin synthesis.**

Parsley suspension cell; Signal transduction; Elicitor-induced $Ca^{2+}$ uptake; $K^+$ release; Coumarin synthesis; Protein kinase inhibitor

## 1. INTRODUCTION

Pathogen-induced formation of toxic secondary metabolites ('phytoalexins') is a well-known defense reaction in higher plants [1,2]. This reaction can be experimentally triggered by substances of microbial origin ('elicitors'), which are thought to represent the chemical basis for recognition of potential pathogens by plants. Regulation of phytoalexin production involves transcriptional activation of genes leading to increased synthesis of phytoalexin biosynthetic enzymes, but little is known of the signal transduction mechanism for enzyme induction [1–3]. We have here used suspension-cultured parsley cells, which respond to a glycoprotein elicitor present in cell wall extracts of the fungus *Phytophtora megasperma* f.sp. *glycinea* to produce coumarin derivatives as phytoalexins [1–5].

After in vivo labeling of parsley cells with [$^{32}$P]orthophosphate, the elicitor induces a rapid and transient increase in the labeling of discrete phosphoproteins [6]. The function of these phosphoproteins is unknown; however, the kinetics of labeling are indicative of their role in signal transduction. Recent experiments with parsley cells suspended in diluted growth medium show that efflux of $K^+$, an external alkalinization and an increased uptake of $Ca^{2+}$ into the cells start a few minutes after elicitor addition and last for about 20 min (C. Colling, K. Hahlbrock, H. Kauss and D. Scheel, unpublished). Similar changes in $K^+$ flux and pH have been previously observed for other elicitor-defense responses [3]. Both effects appear to be intimately connected, but the mechanism involved is controversial. Suggested mechanisms include a $K^+/H^+$ exchange process, involvement of the $H^+$-ATPase in addition to $K^+$ channels or simply relate the observed alkalinization to an incomplete anion balance and the laws of electroneutrality (for citations see ref. 3).

Here we have used K-252a, an alkaloid antibiotic from *Nocardiopsis* sp., which is a potent inhibitor of various animal protein kinases [7]. Recently, this substance has been shown to inhibit the protein kinase activity in microsomal preparations from tomato cells with a $K_i$ of about 15 nM [8]. It also inhibits the elicitor-induced increase in phenylalanine ammonia-lyase and ethylene biosynthesis in the same cells with half-maximal effects at 0.1 μM [8]. The present results may link in future the early and late physiological elicitor responses with the observed biochemical changes in phosphoproteins [6], and thus help to further elucidate the signal transduction pathway.

*Correspondence address:* H. Kauss, FB Biologie, Universität Kaiserslautern, Postfach 3049, D-6750 Kaiserslautern, Germany

## 2. MATERIALS AND METHODS

A cell culture of *Petroselinum crispum* was grown as described [9] for 3 days and contained 60 to 70 mg cells per ml. The cells (60 mg · ml$^{-1}$) were suspended in 1 mM Bistris/Mes buffer, pH 5.8, containing 4% (v/v) fresh B5 growth medium and 3% (w/v) sucrose.

Coumarin synthesis was studied using aliquots of 2 ml [9]. The inhibitor was added in 40 μl of 10% (v/v) dimethyl sulfoxide and the elicitor in 12 μl of water 0.5 h after K-252a. Coumarins in the 1:100- or 1:1000-diluted supernatant were determined fluorometrically after 24 h [9]. A relative fluorescence of 1 indicates that a 1:100 dilution resulted in 100 scale units when the sensitivity of the Kontron SFM25 spectrofluorometer was set at 432 V ($\lambda_{ex}$ = 335 nm, $\lambda_{em}$ = 398 nm). The results are the means of two parallel samples.

To determine the uptake of $^{45}Ca^{2+}$ into a cellular pool not readily exchangeable with external $La^{3+}$, 0.3 ml aliquots of cell suspension were preincubated in round-bottomed plastic vials for 1 h with $^{45}Ca^{2+}$ (about $6 \times 10^4$ cpm, added in 10 μl suspension medium). K-252a was added shortly before elicitor (zero time). At the times indicated, the

Table I

Influence of protein kinase inhibitor K-252a on elicitor-induced $K^+$ efflux, external alkalinization and $^{45}Ca^{2+}$ uptake into parsley cells

| Additions | Final conc. | External changes in $K^+$ (mM) | External changes in Ph | Uptake of $Ca^{2+}$ ($\times 10^{-3}$ cpm) |
|---|---|---|---|---|
| Elicitor | 20 $\mu g \cdot ml^{-1}$ | + 0.14 | + 0.06 | + 1.39 |
| Elicitor | 60 $\mu g \cdot ml^{-1}$ | + 0.40 | + 0.13 | + 2.65 |
| K-252a | 0.2 $\mu M$ | −0.20 | −0.09 | 0.0 |
| K-252a + elicitor | 0.2 $\mu M$ + 20 $\mu g \cdot ml^{-1}$ | −0.20 (0) | −0.07 (33) | + 0.21 (15) |
| K-252a + elicitor | 0.2 $\mu M$ + 60 $\mu g \cdot ml^{-1}$ | −0.10 (25) | −0.02 (54) | + 1.22 (46) |

Values refer to 1 h incubation at which time control samples (containing 0.2%, v/v, dimethyl sulfoxide) exhibited 2.50 ± 0.01 mM $K^+$, pH 6.35 ± 0.01 and 2331 ± 129 cpm $^{45}Ca^{2+}$ in the cells from a 0.3 ml suspension. These values were subtracted from the treated sample values. The SD in control and test samples were approx. equal. The numbers in parentheses are the % of the elicitor-induced ionic changes remaining unaffected by K-252a. Variability of $^{45}Ca^{2+}$ uptake induced by 0.2 $\mu M$ K-252a alone (see Fig. 2) is discussed in the text.

samples were supplied with 2 ml of ice-cold 2 mM $LaCl_3$ and after at least 1.5 h at 0° washed 3 times with 5 ml of cold 2 mM $LaCl_3$ on GF/A glass microfiber filters. The filters were placed in 3 ml of water-miscible Quicksafe cocktail (Zinsser, Frankfurt, FRG) overnight, the vials gently shaken next morning and counted.

$K^+$ in 0.4 ml samples (Table I) was determined with a $K^+$-selective electrode (Metrohm) using 3 parallel samples which were centrifuged (12 000 × g, 2 min), and the supernatant 0.25 ml was then added to 5 ml of 0.12 M NaCl. The pH values for Table I were determined with a combined glass microelectrode (Metrohm) using 3 samples of 0.4 ml each. For greater cell suspension volumes (12.5 ml, Fig. 3), the above ion-selective electrodes were directly inserted into the shaken cell suspensions.

Inhibitor K-252a was purchased from Fluka and solubilized in dimethyl sulfoxide to give a 1 mM stock solution from which a 1:10 dilution with water was freshly prepared each day. Crude Pmg-elicitor was prepared as described [4,5].

## 3. RESULTS

Within a few minutes after addition to a parsley cell suspension, elicitor induced $K^+$ release and external alkalinization (Fig. 1). This process was greatly inhibited when 0.2 $\mu M$ K-252a was added shortly before the elicitor. When K-252a was added at a time subsequent to elicitor addition (Fig. 1, dashed lines), an immediate decrease of the rate of $K^+$ release and external alkalinization occurred. Subsequently, an acidification and $K^+$ uptake were observed. This latter effect was induced by the inhibitor alone after a lag-time of about 5 min (Fig. 1). Similar effects on $K^+$ flux and pH were seen when the experiments were performed in a small sample volume, which was for economic and technical reasons better suited for the study of $^{45}Ca^{2+}$ uptake (Table I, Fig. 2). Elicitor induced $^{45}Ca^{2+}$ uptake was inhibited by K-252a. K-252a alone induced some $^{45}Ca^{2+}$ uptake, with some variation between individual experiments. Thus in some experiments (e.g. Table I), no $^{45}Ca^{2+}$ uptake was observed at 0.2 $\mu M$ K-252a while in other experiments (e.g. Fig. 2) 0.2 $\mu M$ induced a considerable $^{45}Ca^{2+}$ influx. However, the inhibition of elicitor-induced $^{45}Ca^{2+}$ uptake by K-252a occurred at lower concentrations than did the K-252a-induced $^{45}Ca^{2+}$ uptake, and thus the two effects of K-252a could be distinguished.

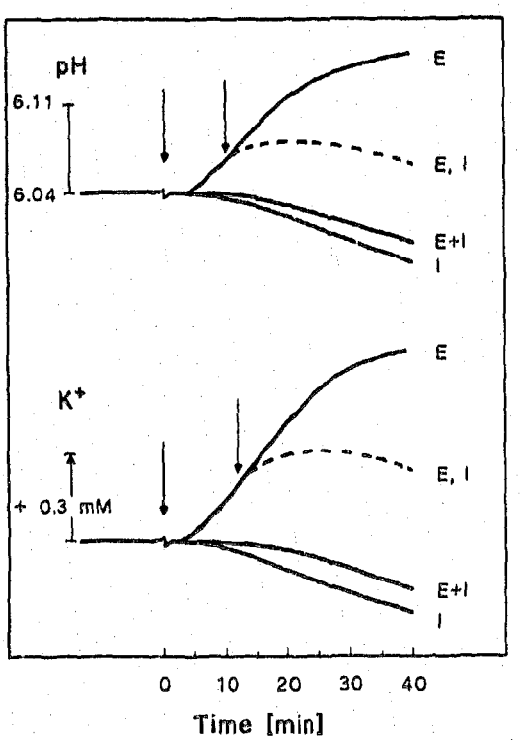


Fig. 1. Influence of protein kinase inhibitor K-252a (I, 0.2 $\mu M$) on changes of pH and the concentration of $K^+$ in parsley cell suspensions treated with elicitor (E, 60 $\mu g \cdot ml^{-1}$). For the solid lines, E, I or E + I were added at the first arrow. For the dashed line, E was added at the first and I at the second arrow. All samples without I received 0.2%, v/v, dimethyl sulfoxide at the appropriate times.

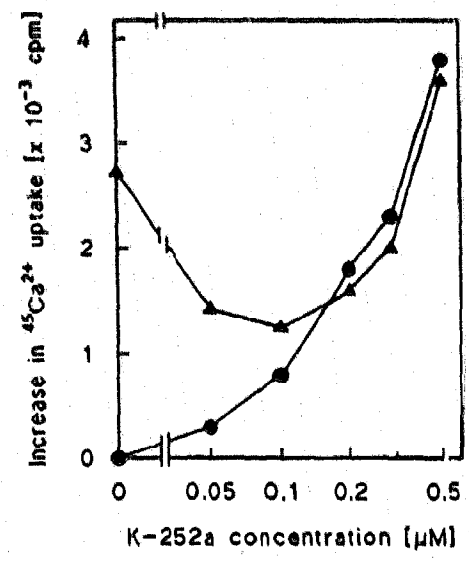


Fig. 2. Influence of protein kinase inhibitor K-252a on the elicitor-induced increase in $^{45}Ca^{2+}$ uptake (▲), and the induction of $^{45}Ca^{2+}$ uptake by K-252a without elicitor (●). The elicitor (60 μg · ml$^{-1}$) was added about 1 min after K-252a, all control samples receiving 0.2%, v/v, dimethyl sulfoxide. Experimental details as for Table I but with 0.5 h incubation after elicitor addition. At this time the control cells in 0.3 ml samples contained $3.9 \times 10^3$ cpm $^{45}Ca^{2+}$, a value subtracted from all treated samples. For comparison with Table I see text.

Preincubation of the cells for 30 min with K-252a greatly increased the induction of coumarin synthesis (Fig. 3). It was affirmed that the $K^+$ release induced under this condition by the elicitor was also inhibited by K-252a. For instance, in cells treated for 30 min with 0.2 μM K-252a the elicited $K^+$ release was reduced to about one-third (data not shown). Given alone, K-252a did

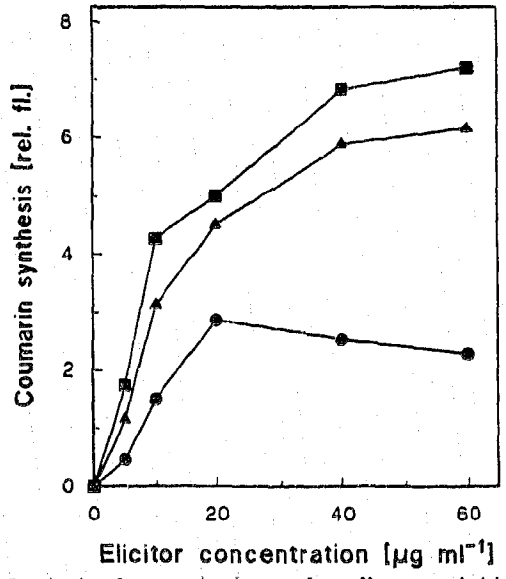


Fig. 3. Synthesis of coumarins by parsley cells suspended in diluted growth medium and induced by various elicitor concentrations in the absence (●) or presence of 0.15 μM (▲) or 0.2 μM (■) K-252a. The inhibitor or 0.2%, v/v, dimethyl sulfoxide in control cells (●) were added 0.5 h before the elicitor.

not elicit coumarin synthesis (Fig. 3). Similar results were found for cells remaining in spent growth medium, the usual protocol in such studies [4-6]. For instance, 40 μg · ml$^{-1}$ elicitor induced 4.3 (rel. fl.) coumarin production which was increased to 8.5 (rel. fl.) by 0.2 μM K-252a. In the undiluted growth medium, $^{45}Ca^{2+}$ uptake induced by 0.2 μM K-252a alone was observed in all experiments and was always higher than the $^{45}Ca^{2+}$ uptake induced by 60 μg · ml$^{-1}$ elicitor. Nevertheless, K-252a given alone in concentrations up to 1 μM again induced no coumarin synthesis (results not shown).

## 4. DISCUSSION

K-252a is a potent inhibitor of protein kinase activity present in microsomal membranes from tomato suspension cells [8]. It appears very likely, therefore, that the effects caused by low concentrations of this inhibitor in parsley cells are also due to inhibition of protein kinase. The inhibitor rapidly affects the elicitor-induced $K^+$ release and associated external alkalinization (Fig. 1, Table I) and the elicited increase in the uptake of $^{45}Ca^{2+}$ into the cells (Fig. 2, Table I). Both $K^+$ and $Ca^{2+}$ most likely move along their gradients across the plasma membrane. Inhibition of such an ion-movement at rather low concentrations of K-252a is unlikely, due to unspecific influence on membrane permeability caused by the hydrophobic nature of the inhibitor [7]. The results, therefore, suggest that transport proteins are involved that are regulated by phosphorylation-dephosphorylation. Elicitor and K-252a appear to influence the regulation of these transport proteins, although in an opposing manner. The nature of the transport involved remains unknown. As K-252a inhibits the elicitor-induced $K^+$ release and also induces $K^+$ uptake (Fig. 1, Table I), $K^+$ channels [11] and active $K^+$ transport must be considered in future attempts to further characterize biophysically the inhibitor effects. The multiple responses observed on $^{45}Ca^{2+}$ uptake into a cellular pool not readily exchangeable with external $La^{3+}$ (Fig. 2) also suggest that several components of the $Ca^{2+}$ transport system might be affected by K-252a.

The increase in coumarin synthesis due to the preincubation of the parsley cells with K-252a (Fig. 3) again suggests that protein phosphorylation is involved in signal transduction between elicitor-binding and gene activation which is known to be a major regulatory feature in this system [1,2,6]. K-252a, given alone, did not induce coumarin synthesis (Fig. 3) but was able to cause $^{45}Ca^{2+}$ uptake (Fig. 2). Therefore, the influx of $Ca^{2+}$ alone is not sufficient for coumarin synthesis. The same conclusion can be drawn from the observation that the $Ca^{2+}$ ionophore A-23187 up to 100 μM does not induce coumarin synthesis in parsley cells (data not shown). These observations are in contrast to reports, e.g. with soybean cells, where A-23187

stimulates some glyceollin accumulation [12]. Since the inhibition of protein kinase enhances coumarin synthesis (Fig. 3), a decrease in the phosphorylation status of a critical phosphoprotein might provide a further signal. This is in apparent contradiction to the observation of elicitor-induced increases in the phosphorylation of some unknown proteins in the same parsley culture [6]. Our observation in parsley fits better with the marked decrease in the phosphorylation of some peptides which has been observed in soybean cells challenged with a glucan-type elicitor [13]. Although $Ca^{2+}$ obviously is not the only signal in parsley, it appears to play a physiological role in coumarin induction since a considerable decrease both in elicitor-stimulated [$^{32}$P]phosphoprotein formation and in coumarin synthesis is observed in cells suspended in $Ca^{2+}$-depleted culture medium [6], and coumarin synthesis is fully inhibited by 0.25 mM $La^{3+}$ (data not shown).

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