K-252a inhibits the response of tomato cells to fungal elicitors in vivo and their microsomal protein kinase in vitro

Debora G. Grosskopf, Georg Felix and Thomas Boller

Friedrich Miescher-Institute, Basel, Switzerland

Received 30 August 1990; revised version received 21 September 1990

Two characteristic responses of plant cells to fungal elicitors, induction of phenylalanine ammonia-lyase activity and of ethylene biosynthesis, were studied in suspension-cultured tomato cells. Induction of both responses was completely blocked by 500 nM K-252a, a known inhibitor of mammalian protein kinases. About 100 nM K-252a caused half-maximal inhibition. In vitro, K-252a inhibited protein kinase activity in microsomal preparations from tomato cells. Inhibition was competitive with respect to ATP and had a K_i of about 15 nM. Thus, protein kinases sensitive to K-252a occur in plants and might be important for the plant's response to fungal elicitors.

Ethylene biosynthesis; Phenylalanine ammonia-lyase; K-252a; Protein kinase; Elicitor; Tomato

1. INTRODUCTION

Plant cells react to chemical stimuli derived from fungi, so-called elicitors, with a multifaceted defense response [1]. A frequently studied element of this response is the induction of phenylalanine ammonia-lyase (PAL), due to transcriptional activation [1,2]. Among the numerous other responses to elicitors, a rapid induction of ethylene biosynthesis is particularly easy to measure [3,4]. We have used this parameter to screen for substances which interfere with the plant's response to elicitors. Here we report that submicromolar concentrations of the microbial metabolite K-252a prevent induction of ethylene biosynthesis and of PAL by fungal elicitors. K-252a, a glycosylated indole carbazole alkaloid resembling staurosporine, is a potent inhibitor of several protein kinases in mammalian systems [5]. We also show that K-252a is a potent inhibitor of protein kinase activity in tomato microsomes in vitro, suggesting that K-252a probably acts on plant cells, as on animal cells, through inhibition of protein kinase activities.

2. EXPERIMENTAL

2.1. *Plant cells and elicitor treatments*

A cell suspension culture was prepared from a tomato callus, Msk8 [6], kindly provided by Dr M. Koornneef (Wageningen, The Netherlands). It was grown at 27°C in a Murashige-Skoog-type liquid medium supplemented with 5 μM 1-naphthylacetic acid, 1 μM 6-benzyladenine, and vitamins as described [7], and subcultured at three-week intervals. For elicitor treatments, cells (ca 0.2 g fresh weight in 2 ml medium) were mixed with 20 μl of appropriate dilutions of the elicitors or water (controls), enclosed in 6-ml-reagent tubes capped with a rubber septum, placed horizontally on a shaker and incubated at room temperature.

2.2. *Determination of ethylene production and PAL activity*

After 4 h of incubation, a gas sample (1 ml) was withdrawn from the reagent tubes and assayed for ethylene by gas chromatography [3]. Immediately after, cells were washed on a filter, frozen, homogenized in 100 mM Tris-HCl, pH 8.8, containing 5 mM 2-mercaptoethanol, and used for PAL assays [8].

2.3. *Pulse-labelling of tomato cells with [^{35}S]amino acids*

Appropriately treated cells (0.05 g in 0.2 ml medium) were incubated in Eppendorf tubes for 180 min, pulse-labelled with 0.4 MBq [^{35}S]methionine and cysteine (Tran^{35}S-label, ICN Biomedicals) for 20 min, washed three times with 0.4 ml 1 mM unlabelled methionine, and frozen in liquid nitrogen. After thawing, cells were homogenized in 0.1 ml 100 mM Tris-HCl buffer, pH 7.6, containing 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol, and centrifuged at 3000 × g for 10 min. Aliquots of the supernatants corresponding to 4 kBq were subjected to SDS-PAGE on 14% polyacrylamide gels [9], prepared for fluorography using En^{3}hance (Du Pont), dried and exposed to X-ray film.

2.4. *Assay of microsomal protein kinase*

Microsomes were prepared as described [10], layered on 0.5 M sucrose and collected by centrifugation (80000 × g, 120 min). The pellet was resuspended in 25 mM Tris-Mes buffer, pH 7.8, containing 1 mM dithiothreitol, and stored at − 80°C until use. Mixtures for protein kinase assay contained 50 mM Tris-Mes, pH 7.8, 1 mM $MgCl_2$, 1 mM dithiothreitol, 60 kBq [γ-^{32}P]ATP at different concentrations, K-252a where appropriate, and 20 μg histone-III (Sigma, type III-S) in a total volume of 50 μl. The reaction was started by addition of 10 μl microsomes containing 2 μg protein. After 5 min at 25°C, duplicate samples (10 μl) were transferred onto pieces of phosphocellulose paper (Whatman P81) and immediately plunged into 5% (v/v) phosphoric acid. Radioactivity bound to the phosphocellulose was determined as in [11], taking blanks lacking enzyme and samples stopped at zero time into account.

Correspondence address: T. Boller, Friedrich Miescher-Institute, POB 2543, CH-4002 Basel, Switzerland

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

2.5. *Elicitors and chemicals*

Pmg elicitor, a partial acid hydrolysate from cell walls of *Phytophthora megasperma* pv. *glycinea*, was kindly provided by Dr J. Ebel (Freiburg i. Br., FRG). Yeast extract elicitor was isolated from yeast extract (Difco) by dialysis and partial purification on a DEAE-Trisacryl column [4]. A preparation of xylanase from *Trichoderma viride* was purchased from Fluka. Stock solutions of K-252a (Calbiochem) were prepared in dimethylsulfoxide. Control experiments showed that dimethylsulfoxide at concentrations up to 1% did not change any of the elicitor effects observed (data not shown).

3. RESULTS

Three types of fungal elicitors, a glycopeptide fraction isolated from yeast extract, a glucan-rich cell wall hydrolysate from the soybean pathogen, *Phytophthora megasperma* pv. *glycinea*, and a xylanase from the saprophyte *Trichoderma viride*, were potent inducers of ethylene biosynthesis and PAL activity in tomato cells. At concentrations of 10 $\mu g \cdot ml^{-1}$, all three stimulated ethylene biosynthesis fivefold to tenfold within the first 4 h compared to the control treatment (Table I). All elicitors induced PAL activity about 4-fold during the same interval. The control treatment, which consisted in shaking cells in assay tubes without elicitor, also caused an increase in the rate of ethylene biosynthesis and PAL activity, indicating that the assay conditions themselves represented a stress to the cells (Table I).

K-252a (500 nM), added 5 min before the elicitors, completely blocked induction of both ethylene biosynthesis and PAL activity (Table I). It also prevented the increase in ethylene production and PAL activity observed in control cells shaken without elicitors, indicating that K-252a inhibited the general stress-induced response as well. A dose-response curve showed that about 100 nM K-252a were needed to reduce elicitor-induced ethylene biosynthesis by 50% (Fig. 1).

Cells incubated with or without elicitor and/or K-252a for 3 h and then pulse-labelled with [^{35}S]methionine for 20 min incorporated similar amounts of radioactivity into TCA-precipitable material. Fluorograms of SDS-PAGE gels loaded with

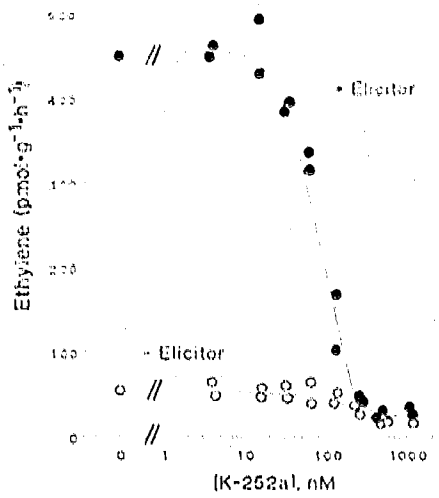

Fig.1. Dose-response curve for the inhibition of elicitor-stimulated ethylene production by K-252a. Cells were treated with different concentrations of K-252a and with 10 $\mu g \cdot ml^{-1}$ yeast extract elicitor (closed circles) or with water (open circles), and incubated for 4 h.

total cell extracts (Fig. 2) showed a strong increase in radioactivity of several major polypeptides and the disappearance of at least one other in elicitor-treated cells (lane B) as compared to control cells (lane A). Treatment with 500 nM K-252a did not inhibit protein synthesis in general. Although it caused some minor changes in the basal pattern of protein synthesis (compare lane A and C), its major effect was to prevent the changes in the pattern of newly synthesized proteins in elicitor-treated cells (compare lanes B and D).

Protein kinase activity was examined in microsomal preparations obtained from tomato cells, using histone-III as an exogenous substrate. The specific activity of protein kinase was similar in microsomes from untreated and elicitor-treated cells (data not shown). Microsomal protein kinase activity, which had a V_{max} of about 25 pkat $\cdot$ mg^{-1} protein and a K_m of ca 100 μM

Table I

Effect of K-2522a on induction of ethylene biosynthesis and phenylalanine ammonia-lyase (PAL) activity by various elicitors. Data represent means and standard deviation of 4 replicate samples

Treatment[a]	Ethylene biosynthesis ($pmol \cdot g^{-1} \cdot h^{-1}$)		PAL activity ($pkat \cdot g^{-1}$)	
	− K-252a	+ K-252a	− K-252a	+ K-252a
None [b]	22 ± 9	25 ± 4	≤ 15	23 ± 10
Control (4 h)	107 ± 19	35 ± 8	62 ± 22	≤ 15
Elicitors (10 $\mu g \cdot ml^{-1}$, 4 h)				
Yeast extract elicitor	897 ± 81	37 ± 11	207 ± 49	≤ 15
Pmg-elicitor	948 ± 241	57 ± 16	274 ± 77	18 ± 6
Xylanase	546 ± 74	43 ± 6	245 ± 39	18 ± 6

[a] Cells (2 ml) received either 1 μl dimethylsulfoxide (columns labelled '− K-252a') or 1 μl K-252a in dimethylsulfoxide (columns labelled '+ K-252a') 5 min before addition of elicitors or water (controls)

[b] PAL-activity in cells harvested before treatment and rate of ethylene biosynthesis during the first hour of control treatment

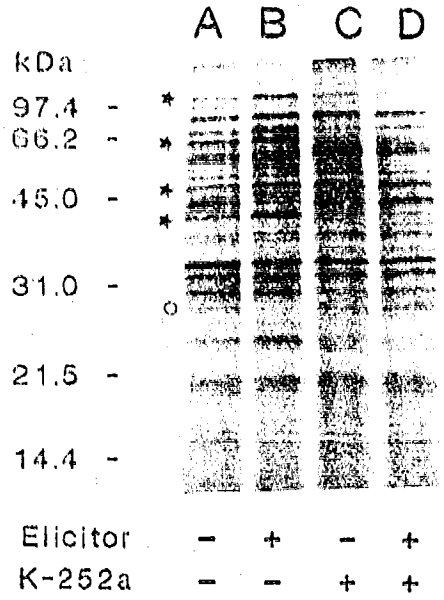

Fig.2. Fluorogram of cell proteins pulse-labelled with [^{35}S]methionine for 20 min after treatments with or without 500 nM K-252a and/or 10 $\mu g \cdot ml^{-1}$ yeast extract elicitor for 3 h. Equal amounts of TCA-insoluble radioactivity, corresponding to similar amounts of total protein (10-15 μl cell extract per lane), were subjected to SDS-PAGE. Major polypeptide bands appearing (★) or disappearing (○) upon elicitor treatment are marked.

for ATP, was strongly inhibited by K-252a. Inhibition was competitive with respect to ATP; the apparent K_i was ca 15 nM (Fig. 3).

4. DISCUSSION

Our data show that K-252a is a highly potent inhibitor of the induction of two characteristic responses of tomato cells to elicitor treatments. K-252a has little effect on protein synthesis in non-elicited cells, indicating that it does not cause gross changes in cell metabolism in general. It also has no direct adverse effects on growth, PAL activity and ethylene biosynthesis (data not shown). However, it does block the changes in protein synthesis, PAL activity and ethylene biosynthesis induced by elicitors (and also by the stress due to the experimental conditions), suggesting that it interferes with some step(s) of the induction process.

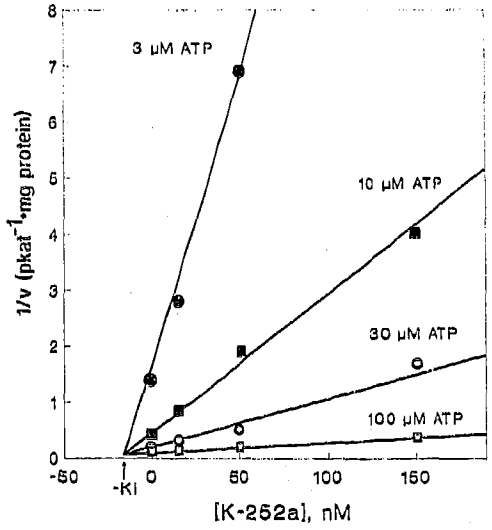

Fig.3. Inhibition of protein kinase in tomato microsomes by K-252a, presented in a Dixon plot.

K-252a is a specific inhibitor of protein kinases in animal systems [5]: it has frequently been used as an inhibitor of responses dependent on protein kinase C but inhibits a variety of other protein kinases as well [5]. Our results show that plant microsomes contain a protein kinase activity that is as sensitive to K-252a as the most sensitive mammalian protein kinases, displaying a K_i of ca 15 nM. At a recent meeting, K-252a has been reported to inhibit a protein kinase from the chromatin of pea nuclei with a K_i of ca 80 nM [12]. Thus, it is likely that K-252a exerts its effects on the elicitor response in plants through inhibition of protein kinase activities, like in animals. This hypothesis is compatible with recent evidence that the pattern of protein phosphorylation is altered in vivo in plant cells responding to elicitors [13].

It is worth noting that in our system, H7 (1-(5-isoquinoline sulfonyl)-2-methylpiperazine), a potent inhibitor of protein kinase C from animals, as well as OAG (1-oleoyl-2-acetyl-*sn*-glycerol) and TPA (tumor-promoting phorbol ester, phorbol-12-myristate-13-acetate), two typical activators of this enzyme [14], had no effect on the basal rate of ethylene production, nor did they affect its induction by elicitors (D.G. Grosskopf et al., in preparation). Thus, we have no evidence for an involvement of protein kinase C, as operationally defined in mammalian systems. The physiological role(s) of protein kinase C homologues in plants that have been characterized at the molecular [15] and biochemical [16–18] level remain to be established. However, plants clearly have protein kinases highly sensitive to K-252a, and we are now studying which of these are important for the induction of the response to elicitors and by which mechanisms they might exert their possible regulatory function.

Acknowledgements: We thank Dr M. Koornneef for supplying the tomato cell line Msk8, Dr J. Ebel for the elicitor preparation from *Phytophthora megasperma*, M. Regenass for expert technical assistance, Th. Leitz for helpful discussions and H.A. Lane, D. Reddy and K. Ballmer for critically reading the manuscript.

REFERENCES

[1] Dixon, R.A. and Lamb, C.J. (1990) Annu. Rev. Plant Physiol. Mol. Biol. 41, 339–367.
[2] Hahlbrock, K. and Scheel, D. (1990) Annu. Rev. Plant Physiol. Mol. Biol. 40, 347–364.

[3] Chappell, J., Hahlbrock, K. and Boller, T. (1984) Planta 161, 475–480.
[4] Spanu, P., Felix, G. and Boller, T. (1990) Plant Physiol., in press.
[5] Ruegg, U.T. and Burgess, G.M. (1989) Trends Pharm. Sci. 10, 218–220.
[6] Koornneef, M., Hanhart, C.J. and Martinelli, L. (1987) Theor. Appl. Genet. 74, 633–641.
[7] Adams, T.L. and Townsend, J.A. (1983) Plant Cell Reports 2, 165–168.
[8] Lamb, C.J., Merritt, T.K. and Butt, V.S. (1979) Biochim. Biophys. Acta 582, 196–212.
[9] Laemmli, U.K. (1970) Nature 227, 680–685.
[10] Gallagher, S.R. and Leonard, R.T. (1982) Plant Physiol. 70, 1335–1340.
[11] Corbin, J.D. and Reimann, E.M. (1974) Methods Enzymol. 38, 287–290.
[12] Li, H. and Roux, S. (1990) Plant Physiol. 93, Suppl. 63.
[13] Dietrich, A., Mayer, J.E. and Hahlbrock, K. (1990) J. Biol. Chem. 265, 6360–6365.
[14] Farago, A. and Nishizuka, Y. (1990) FEBS Lett. 268, 350–354.
[15] Lawton, M.A., Yamamoto, R.T., Hanks, S.K. and Lamb, C.J. (1989) Proc. Natl. Acad. Sci. USA 86, 3140–3144.
[16] Ranjeva, R. and Boudet, A.M. (1987) Annu. Rev. Plant Physiol. 38, 73–93.
[17] Olah, Z., Bogre, L., Lehel, C., Farago, A., Seprodi, J. and Dudits, D. (1989) Plant Mol. Biol. 12, 453–461.
[18] Park, M.-H. and Chae, Q. (1990) Biochem. Biophys. Res. Commun. 169, 1185–1190.