Ergosterol Induces Mobilization of Internal Calcium in Tobacco Cells

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Abstract

As for natural sterols, only ergosterol is recognized very specifically and sensitively (nM) by plants cells. Ergosterol interacts with tobacco suspension cells and trigger pH changes of extracellular medium, oxidative burst and synthesis of phytoalexins. Compared with the responses induced by cryptogein, a proteinaceous elicitor from *Phytophthora* sp., oxidative burst, DpH and phytoalexin accumulation were weaker with ergosterol. Cryptogein stimulated an apparent continuous uptake of external calcium within 40 min, whereas no net uptake of external calcium occurred upon the addition of ergosterol. However, the elicitation with either cryptogein or ergosterol resulted in an increase of the fluorescence of calcium green 1 in cytosol. The use of several inhibitors of calcium channels (La^{3+}, TMB-8, verapamil, ruthenium red, nifedipine) and a protein-kinase inhibitors (staurosporin, NPC-15437, H-89) suggests that the elicitation with ergosterol includes the mobilization of internal calcium stores in vacuoles mediated by IP3 and some protein kinases.

Keywords: elicitor; ergosterol; calcium channels

INTRODUCTION

The molecular mechanism of the interaction between hosts and pathogens is a subject of many studies. Usually, the products of pathogen avirulence genes named elicitors bind to receptors and trigger a signal transduction cascade. The changes of membrane permeability leading to the influx of Ca^{2+} and H^+ and the efflux of Cl^- and K^+ followed by the oxidative burst, synthesis of phytoalexins and the activation of defence genes constitute the response of plants to microorganism attacks. The elicitors are oligosaccharides, peptides or low-molecular substances released from hyphen or plant cell walls. Concerning lipids, GRANADO *et al.* (1995) previously reported that a specific interaction of ergosterol from *Cladosporium fulvum* with tomato cells induces extracellular pH changes.

Calcium channels are involved principally in signal transduction in plant cells. There were characterized in the plasma membrane, endoplasmic reticulum, tonoplast, nuclear and plastid membrane (for review see WHITE (2000)). The presence of calmodulin, Ca^{2+} – dependent protein kinases and Ca^{2+} – stimulated phospholipases indicates that changes in cytosolic free calcium has profound effects on cellular function (TREWAVAS & GILROY 1991).

This paper examines the mechanism of the elicitation of AOS (active oxygen species) production and H^+ transport by ergosterol, a fungal sterol, in tobacco suspension cells using several calcium channel inhibitors and protein kinases inhibitors. We show that the interaction of ergosterol results in the mobilization of intracellular calcium stores in contrast with the proteinaceous elicitor cryptogein, which interacts with receptors, localised on the surface of the plasma membrane.

MATERIAL AND METHODS

Plant cells harvested in exponential phase of growth were filtered, washed and resuspended in 2mM MES

buffer (pH 5.75) containing 175mM mannitol, 0.5mM K_2SO_4 and 0.5mM $CaCl_2$ (elicitation buffer). The concentration of cells was 0.1 g FW/ml. After a 2 h equilibration period, the cells were treated with the elicitors and the inhibitors.

The production of AOS was determined by luminol reaction in BioOrbit 1253 Luminometer. One ml of the cell suspension was added to 250 μl of 150mM MES (pH 6.5) containing 175mM mannitol, 0.5mM K_2SO_4 and 0.5mM $CaCl_2$ and 50 μl of 0.3mM luminol in measuring cell, then luminescence was immediately read. The pH changes were registered every 5 min after the addition of elicitor.

Ca^{2+} uptake was determined by monitoring the variation of $^{45}Ca^{2+}$ amount associated with the cells as described by TAVERNIER *et al.* (1995).

Phytoalexins were extracted from the medium 24 h after elicitor treatment and their GC analysis was performed according to MILAT *et al.* (1991).

The calcium fluxes in tobacco suspension cells were monitored using Calcium Green 1 acetoxymethylester (CG-1), and a Leica TCS 4D confocal microscope equipped with an argon-krypton laser (excitation filter 488 nm, barrier filter LP515). Tobacco cells cultivated for 3 days in the dark were filtered and resuspended in the elicitation buffer as described above (0.1 g FW/ml). After a 2 h equilibration period at 25°C, aliquotes of suspension cells were removed (1 ml). Then, in two of them (replicates), 5 μl of CG-1 (1 μg/ml in DMSO) and 2.5 μl of ergosterol (0.2 mg/ml in DMSO, 1.2μM final concentration) were added at the same time. In two other samples, 5 μl of cryptogein (0.1 mg/ml in H_2O, 50nM final concentration) were added to 1 ml of the suspension cells and CG-1 (5 μl) was added 20 min after the addition of cryptogein. For all assays, the fluorescence of the cells was observed 10 min after the addition of CG-1. Untreated cells were also observed.

RESULTS AND DISCUSSION

Influence of the sterol structure in elicitation of tobacco cells. Tobacco cells were treated with different sterols; ergosterol, 9-dehydroergosterol, ergocalciferol, cholesterol and 7-dehydrocholesterol. Ergocalciferol is a photodecomposition product of ergosterol, 7-dehydrocholesterol with two double bounds differs from ergosterol in having saturated lateral chain with one methyl group and 9-dehydroergosterol has three double bounds. The production of AOS was assessed using 1.2μM of each sterol studied. The interaction of tobacco cells with sterols presents some specific features because only ergosterol and 9-dehydroergosterol were effective, 7-dehydrocholesterol was slightly efficient whereas cholesterol and ergocalciferol were inefficient. This suggests the significance of two double bonds in the ring B and that of ergosterol side chain for the specificity of binding to a possible receptor.

Ergosterol-concentration dependence of the AOS production and pH changes. The production of AOS (10μM hydrogen peroxide) was maximal and saturated with 30nM ergosterol. The effect on pH changes was more noticeable and even at low ergosterol concentration (such as 5nM), a DpH of 0.31 was observed.

Ergosterol-induced synthesis of phytoalexins. Addition of ergosterol to tobacco suspension cells induced the synthesis of capsidiol. The concentration of capsidiol induced by ethanol as a blank was 26.9 μg/g of cell dry weight, whereas that induced by 25nM ergosterol was 302 μg/g. The synthesis of capsidiol increased when the concentration of ergosterol was increased up to 2.5μM in spite of the fact that the AOS synthesis and pH changes were saturated at 25nM ergosterol.

Calcium-distribution changes in tobacco cells visualized by fluorescence confocal microscopy. The cells were incubated with the elicitors for the time necessary to obtain maximal elicitation effect, i.e. 10 and 30 min for ergosterol and cryptogein, respectively. Elicitation with either cryptogein or ergosterol resulted in an increase of the fluorescence of CG-1 in cytosol by comparison with cells incubated without elicitor.

Ergosterol-elicited influx of calcium into tobacco cells. Elicitation of tobacco cells by cryptogein resulted in a massive uptake of $^{45}Ca2^{+}$ whereas no net change in calcium content occurred upon the addition of ergosterol although ergosterol triggered an increase in the cytosolic $Ca2^{+}$ level as shown by confocal microscopy with calcium green 1. However, in the case of cryptogein/tobacco-cell interaction, this longterm accumulation of calcium has been shown to contribute to the reinforcement of cell wall (PAULY *et al.* 1997).

Role of calcium channels in the elicitation of tobacco cells with ergosterol. Several calcium-channel inhibitors were used to analyse the calcium fluxes induced in tobacco cells by the elicitation with ergosterol. The effects of the inhibitors are shown in Table 1. LaCl3, at micromolar concentrations, inhibited both the AOS production and extracellular medium alkalization induced by cryptotein but it only partially inhibited these responses when ergosterol was used as elicitor. Micromolar La^{3+} concentrations inhibit the activity of most channels in the plasma membrane whereas mili-

Table 1. Effect of calcium-channel inhibitors. The results in percents represented with standard error in parantheses (3 independent cultivations) are the mean activities obtained after 15 min of elicitation compared with the non inhibited reaction

Inhibitor	25nM cryptogein		200nM ergosterol	
	AOS (%)	Δ pH (%)	AOS (%)	Δ pH (%)
100μM $LaCl_3$	32 (11)	31 (2)	100 (10)	75 (10)
300μM $LaCl_3$	6 (1)	1.2 (3)	54 (4)	61 (8)
100μM r.red	–	47 (18)	–	87 (6)
200μM r.red	–	22 (7)	–	80 (12)
100μM verapamil	67 (26)	90 (9)	27 (6)	55 (16)
200μM verapamil	56 (21)	91 (14)	30 (8)	48 (20)
50μM TMB-8	96 (2)	92 (4)	33 (7)	32 (2)
100μM TMB-8	97 (4)	97 (8)	26 (11)	24 (4)
100μM nifedipine	–	105 (13)	–	95 (2)
200 μM nifedipine	–	99 (1)	–	91 (1)

molar concentrations are necessary to affect vacuolar channels (PINEROS & TESTER 1997). Nifedipine inhibit voltage dependent calcium channels tomato and wheat plasma membrane and tonoplast (PINEROS & TESTER 1997). It inhibited neither the effect of cryptogein nor ergosterol. Ruthenium red is the inhibitor of voltage-dependent calcium-channels on plasma membrane and cADPR dependent channels on tonoplast (MUIR *et al*. 1997). It had a pronounced effect on the elicitation with cryptogein but it affected only slightly the elicitation with ergosterol. On the other hand, TMB-8, antagonist of IP_3-mediated intracellular calcium release (MUIR *et al*. 1997) did not significantly inhibit AOS synthesis and pH changes induced by cryptogein but it strongly reduced the extracellular alkalization and the AOS synthesis induced by ergosterol. This could suggest that the elicitation with ergosterol does not involve the participation of plasma membrane calcium channels. However, it involves the mobilization of internal calcium stores in vacuoles probably mediated by IP3 and not by cADPR as in the case of abscissic acid (WHITE 2000). Verapamil, inhibitor of plasma membrane and tonoplast voltage-dependent calcium-channels and IP3 dependent calcium channels on tonoplast (PINEROS & TESTER 1997), inhibited pH changes induced by ergosterol but not those induced by cryptogein.

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