Early Elicitor-Induced Events in Chickpea Cells: Functional Links between Oxidative Burst, Sequential Occurrence of Extracellular Alkalinisation and Acidification, K⁺/H⁺ Exchange and Defence-Related Gene Activation

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Elicitation of cultured chickpea (*Cicer arietinum* L.) cells stimulates a signal transduction pathway leading to several rapid responses: (1) oxidative burst, (2) extracellular alkalinisation, (3) extracellular acidification, (4) transient K⁺ efflux, and (5) activation of defence related genes all within 2 hours. Induced genes are encoding acidic and basic chitinases, a thaumatin-like protein and isoflavone reductase. All these elicitor-induced responses are inhibited by the Ser/Thr protein kinase inhibitor staurosporine and the anion channel blocker anthracene-9-carboxylic acid but stimulated by the Ser/Thr protein phosphatase 2A inhibitor cantharidin. The oxidative burst leads to a transient extracellular H_2O_2 accumulation which seems to be preceded by $\cdot O_2^-$ production, indicating dismutation of $\cdot O_2^-$ to H_2O_2. The oxidative burst is accompanied by transient alkalinisation of the culture medium which is followed by long-lasting extracellular acidification. An 80 percent inhibition of the alkalinisation after complete inhibition of the H_2O_2 burst with diphenylene iodonium indicates that the elicitor induced increase of extracellular pH is mainly based on a proton consumption for $\cdot O_2^-$ dismutation. A simultaneous deactivation of the plasma membrane H⁺-ATPase during oxidative burst and extracellular alkalinisation is also suggested. The elicitor-stimulated extracellular acidification is inhibited by the plasma membrane H⁺-ATPase inhibitor N, N′-dicyclohexylcarbodiimide assuming a reactivation of the H⁺-ATPase 25 min after elicitation. Extracellular acidification seems not to be necessary for elicitor-induced activation of defence related genes. Opposite modulation of K⁺ and proton fluxes after elicitation and/or treatment with the H⁺-ATPase effectors fusicoccin or N, N′-dicyclohexylcarbodiimide indicate that the elicitor induced transient K⁺ efflux is regulated by a K⁺/H⁺ exchange reaction.

Introduction

In the last years an array of early transcription-independent responses of plant cells to microbial infection or elicitation have been detected and characterised. One of the earliest observable traits of plant cells is the oxidative burst (reviewed by Lamb and Dixon, 1997) which is generally defined as a rapidly stimulated production of reactive oxygen species (ROS), including superoxide anion ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$) and hydrogen peroxide (H_2O_2). Oxidative burst is often accompanied by other early defence responses: (1) peroxidative insolubilisation of plant cell wall proteins (Otte and Barz, 1996), (2) changes in extracellular pH values (Felix *et al.*, 1993), (3) ion fluxes (Nürnberger *et al.*, 1994) and (4) changes in protein phosphorylation patterns (Felix *et al.*, 1993). After elicitation a reversible induction of plasma membrane (PM) H⁺-ATPase activity by phosphorylation has also been reported (Vera-Estrella *et al.*, 1994). Well documented transcription-dependent responses of intact plants and cell cultures after pathogen attack or elicitation are the formation of antimicrobial phytoalexins and pathogenesis-related (PR) proteins. Co-ordinated activation of genes encoding enzymes of the long biosynthetic

pathways of phytoalexins (reviewed by Barz, 1997) and the heterogeneous family of PR proteins have been found to occur (van Loon, 1999).

In suspension cells of chickpea elicitation causes an oxidative burst (Otte and Barz, 1996) which is accompanied by other early defence responses: (1) the rapid peroxidative cross-linking of a cell wall located extensin-like and a proline-rich protein, driven by elicitor-induced H_2O_2 (Otte and Barz, 1996, 2000) and (2) changes in extracellular pH values (Pachten and Barz, 1999). Moreover, (3) the possible involvement of ion channels as part of a corresponding signal transduction chain and (4) the dependency of the elicitor response on protein kinase and protein phosphatase activities have been reported (Otte and Barz, 1996; Pachten and Barz, 1999). Comparable to plant-pathogen interaction in cell suspension cultures phytoalexin formation and accumulation of PR proteins can be elicited by treatment with fungal elicitors (Kessmann *et al.*, 1988; Vogelsang and Barz, 1993). Using chickpea cell suspension cultures we have now investigated in more detail the elicitor-induced sequential occurrence of ROS-generation, K^+ fluxes, and extracellular pH changes, respectively, as well as the involvement of the PM H$^+$-ATPase activity in the elicitation response. The measurements also include accumulated amounts of mRNAs of defence-related genes. The causal links between these various reactions are discussed.

Materials and Methods

Plant cell culture

Cell suspension cultures of chickpea (*Cicer arietinum* L., cultivar ILC 3279) were grown as previously described (Kessmann *et al.*, 1988). Three days after subculturing cells were gently separated from the culture medium, washed and resuspended in experiment medium (20% modified culture medium without vitamins and exchange of potassium by sodium salts, $[K^+] = 2.6$ mg l^{-1}, 1% sucrose, pH 5.9). Cells were preincubated for 2 h on a rotary shaker (100 rpm) at a final density of 75 mg FW ml^{-1} before experimental use.

Elicitor

The preparation of *Phytophthora sojae* (*Pmg*) elicitor was performed according to Ayers *et al.*

(1976). The lyophilised elicitor preparation was dissolved in distilled water and autoclaved before use.

Assay for H_2O_2 generation

H_2O_2 was assayed by luminol-dependent chemiluminescence according to Warm and Laties (1982). Two hundred µl-aliquots of the cell suspensions were added to 700 µl potassium phosphate buffer (50 mM, pH 7.9), slightly mixed in a cuvette and directly transferred into a luminometer (Lumat LB 9501/16, Berthold, Germany). Before light detection (10 s-intervals) 100 µl luminol (1.21 mM) and 100 µl $K_3[Fe(CN)_6]$ (14 mM; catalyst) were automatically injected.

Assay for ·O$_2^-$-generation

·O$_2^-$ was assayed by lucigenin-dependent chemiluminescence according to Auh and Murphy (1995). Two hundred µl-aliquots of the cell suspensions were added to 800 µl glycine NaOH buffer (100 mM; pH 9.0; 1 mM EDTA), slightly mixed and directly transferred into the luminometer (see above). Before light detection 100 µl lucigenin (1.21 mM) was automatically injected. Light emission was recorded as the mean of six subsequent 10-s intervals.

Measurement of extracellular K^+ concentration

Three ml-aliquots of the cell suspensions were centrifuged at $3100 \times g$ for 2 min. Cells were separated and extracellular K^+ concentration was measured in the supernatant by atom emission spectroscopy at 766.5 nm (atom absorption spectrometer Unicam 939).

Measurement of extracellular pH

The extracellular pH in suspension cultures was continuously measured with a H$^+$ sensitive microelectrode (Ingold).

Viability test

The viability of suspension cells was determined using the fluorescein diacetate method according to Widholm (1972).

Northern blot analysis of gene expression

Total RNA was extracted from the cells using the single step guanidinium thiocyanate method (Chomszynski and Sacchi, 1987), then phenol extracted and stored at −80 °C. Twenty μg RNA were electrophoresed on a 1% denaturing agarose-formaldehyde gel. The RNA was capillary transferred onto a positively charged nylon membrane (Nytran-Plus, Schleicher & Schuell, Germany) with 20× SSPE and hybridized over night at high stringency in 5× SSC, 1% *N*-lauroyl sarcosine, 0.02% SDS, 2% blocking reagent (Boehringer, Germany) and 50% formamide at 68 °C. Digoxygenin-UTP-labelled (Boehringer) antisense RNA-probes were obtained by *in vitro* transcription of cDNAs of IFR, PR-8a, PR-5b and BC (Ichinose *et al.*, 2000) from chickpea with T7-RNA-Polymerase (Stratagene, La Jolla, USA). Northern blots were developed according to the manufacturer's instructions and washed at least twice at 68 °C for 20 min in 0.1 × SSC, 0,1 × SDS. Signals were detected by incubation with chemiluminescence substrate CDP-Star after 1−5 min exposition to X-ray film. Equal loading of RNA was demonstrated by hybridising with an RNA probe of chickpea ubiquitin (Ubi) mRNA (Ichinose *et al.*, 2000).

Results

Elicitation of ROS formation

A cell wall elicitor prepared from *Phytophthora sojae* (*Pmg* elicitor; 50 μg ml^{-1} routinely used) induced in chickpea cell suspensions a transient accumulation of extracellular H_2O_2 which reached a maximum of about 23 μM after 30 min and then decreased to the basic level of unelicited cells (Fig. 1A). During a subsequent time period of 8 h elicited cells showed no second release of H_2O_2 (Fig. 1B). As had been shown for yeast elicitor in former experiments (Otte and Barz, 1996), the *Pmg* elicitor had essentially the same effects on H_2O_2 production in chickpea cells of the same batch, but a sixty times smaller amount of mass of the *Pmg* elicitor was sufficient for the same degree of stimulation.

The oxidative burst depends on protein phosphorylation. Therefore the protein kinase inhibitor staurosporine (Otte and Barz, 1996) prevented the elicitor effect (Fig. 1A; IC_{50}: 0.2 μM) whereas the protein phosphatase inhibitor cantharidin accelerated, potentiated and extended the elicited H_2O_2 release (Otte and Barz, 1996). With a delay of 30 min cantharidin (10 μM; EC_{50} 7.5 μM) even stimulated a several hours-lasting H_2O_2 burst in
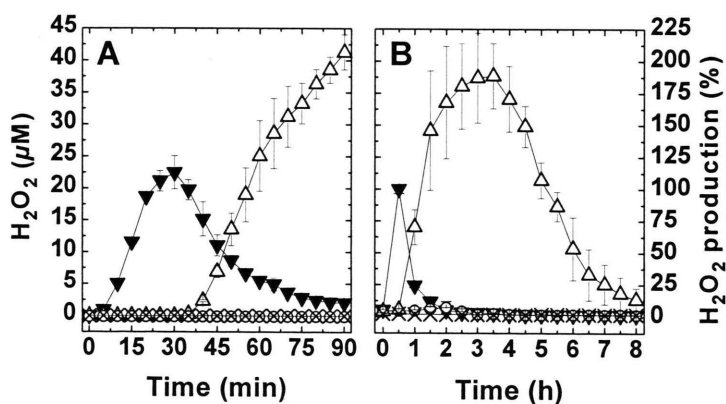
Fig. 1. Short (A) and long term (B) courses of H_2O_2 production in chickpea cell suspensions in response to elicitation and treatment with different effectors.
At time "zero" distilled water (×), *Pmg* elicitor (50 μg ml^{-1}, ▼) or cantharidin (10 μM, △) were separately added to the cell suspensions. Staurosporine (1 μM) was applied 10 min prior to *Pmg* elicitation (○). Every 5 min (A) or 30 min (B) aliquots were withdrawn from the cell suspensions and the H_2O_2 concentration was determined by a luminol chemiluminescence assay (see "Materials and Methods"). B: The maximum concentration of H_2O_2 which accumulated in response to *Pmg* elicitor treatment was defined as 100%. All values represent the means ± standard error of three independent experiments each.

the absence of any elicitor (Fig. 1). As had been shown for yeast elicitor (Otte and Barz, 1996) pre-incubation of cells with low concentrations of DPI (IC$_{50}$: 0.25 μM), known as a suicide flavoprotein inhibitor of the neutrophil PM NADPH oxidase (O'Donnell *et al.*, 1993) completely inhibited the H$_2$O$_2$ accumulation induced by *Pmg* elicitor (Table I). The oxidative burst was also inhibited by the anion channel blockers A-9-C and niflumene acid (Schroeder *et al.*, 1993), the phospholipase C inhibitor neomycin (McDonald and Mamrack, 1995) as well as by the G-protein modulators suramin (Nakajima *et al.*, 1991) and mastoparan (Higashijima *et al.*, 1990). Chelation of Ca^{2+} in the medium with EGTA (3 mM) or application of the Ca^{2+} channel inhibitor verapamil (Piñeros and Tester, 1997) had no clear effect on the generation of an oxidative burst. Table I summarises the effects of the aforementioned inhibitors on H$_2$O$_2$ generation. The Ca^{2+} selective ionophore A23187 (Stäb and Ebel, 1987) was not able to generate an oxidative burst in concentrations of 10–60 μM (data not shown).

Successful measurements of lucigenin-dependent chemiluminescence indicated that the generation of ·O$_2$$^-$ preceded the H$_2$O$_2$ burst (Fig. 2). When diethyldithiocarbamate (DDC; 1 mM), a chelator of copper ions and an inhibitor of the Cu/Zn SOD (Auh and Murphy, 1995) was added to unelicited suspension cells an accumulation of ·O$_2$$^-$ could also be measured. This ·O$_2$$^-$ production was insensitive to catalase and the reaction was increased when DDC was applied together with *Pmg* elicitor. DDC had also the ability to inhibit the elicitor-induced H$_2$O$_2$ burst to control level (Fig. 7). These data indicate a substantial dismutation of ·O$_2$$^-$ to H$_2$O$_2$ via SOD. Surprisingly, only very high amounts of the NADPH oxidase inhibitor DPI (20 μM) had suppressing effects on the elicited ·O$_2$$^-$ generation.

The significance of the data obtained with the various effector and inhibitor compounds also depends on an unaltered viability of the treated cells. From all substances used in our experiments only DDC (1 mM) led to a striking decrease in cellular viability to 75% after 1 h and 5% after 2 h (data not shown).

Table I. Inhibitory effects of several substances on the *Pmg* elicitor-induced oxidative burst and extracellular pH changes.
EGTA was dissolved in the experiment medium. All other substances were added 10 min prior to elicitor (50 μg ml^{-1}). The inhibitory effects on oxidative burst and extracellular alkalinisation were detected 30 min after elicitation, these on extracellular acidification 120 min after elicitation.

| Substance	Function	Inhibition of oxidative burst	Inhibition of extracellular alkalinisation	Inhibition of extracellular acidification
DPI (5 μM)	NADPH oxidase inhibitor	100% (IC$_{50}$: 0,25 μM)	80%	15%
A-9-C (100 μM)	Cl$^-$ channel blocker	85% (IC$_{50}$: 20 μM)	75%	100%
Niflumene acid (200 μM)	Cl$^-$ channel blocker	85% (IC$_{50}$: 90 μM)	n.d.	n.d.
Neomycin (200 μM)	Phospholipase C inhibitor	85% (IC$_{50}$: 115 μM)	50%	100%
Suramin (100 μM)	G-protein modulator	45% (IC$_{50}$: 110 μM)	70%	75%
Mastoparan (20 μM)	G-protein modulator	90% (IC$_{50}$: 10 μM)	n.d.	n.d.
EGTA (3 mM)	Ca^{2+} chelator	0%	n.d.	n.d.
Verapamil (200 μM)	Ca^{2+} channel inhibitor	10%	30%	0%
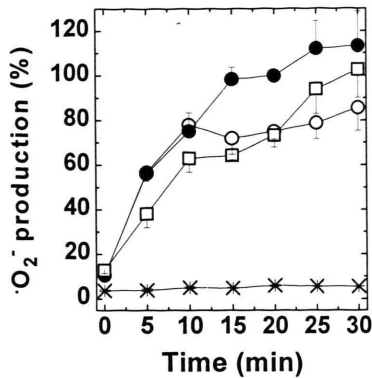
Fig. 2. Time course for $\cdot O_2^-$ accumulation generated by chickpea cells.
At time "zero" DDC (1 mM) was added together either with distilled water (○) or *Pmg* elicitor (50 μg ml⁻¹, ●) to the cell suspensions. Further DDC (1 mM) and *Pmg* elicitor (50 μg ml⁻¹) were both added to the cells at time "zero" after a 50-min pre-treatment with DPI (20 μM, □). The cells were also elicited in the absence of DDC (1 mM, ×). Every 5 min aliquots were withdrawn from the cell suspension and the $\cdot O_2^-$ accumulation were monitored by a lucigenin chemiluminescence assay (see "Materials and Methods"). The average lucigenin chemiluminescence light emission in the assay mixture after a 20-min DDC/*Pmg* elicitor treatment was defined as 100%. Values represent the means ± standard error of three independent experiments each.

Elicitation of extracellular alkalinisation and subsequent acidification

The oxidative burst coincides with an extracellular alkalinisation and this is followed by extracellular acidification. The *Pmg* elicitor first stimulated a rapid and transient alkalinisation of the extracellular medium showing a maximum increase of about 0.4 pH units after 30 min incubation time (Fig. 3A). The pH then decreased again and 2 h after elicitation an acidification with a maximum of 0.5 pH units relative to the initial pH developed (Fig. 3A). This acidification lasted for several hours (Fig. 4). The elicitor stimulated alkalinisation was potentiated and extended by cantharidin (EC₅₀ 7,5 μM) whereas the elicitor stimulated acidification was inhibited or even prevented by this treatment (Table I). Similar to the effect on the oxidative burst cantharidin (10 μM) stimulated a strong and several hours lasting extracellular alkalinisation even in the absence of elicitor (Fig. 3). Preincubation of cells with staurosporine (1 μM) inhibited the elicitor-induced alkalinisation but not the following acidification phase (Fig. 3A and Fig. 4). The elicitor-stimulated alkalinisation was also clearly (80%) but not completely inhibited by
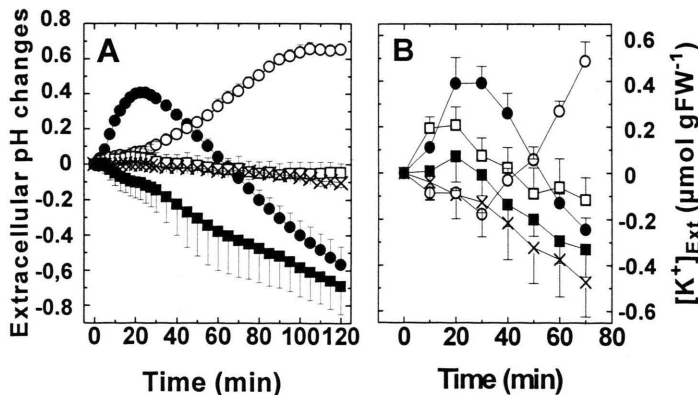

Fig. 3. Time courses for extracellular pH changes (A) and extracellular K⁺ changes (B) in chickpea cell suspensions in response to elicitation and treatment with different effectors.
At time "zero" distilled water (×), *Pmg* elicitor (50 μg ml⁻¹, ●), cantharidin (10 μM, ○) or staurosporine (1 μM, □) were separately added to the cell suspensions. Further staurosporine (1 μM) was added 10 min prior to *Pmg* elicitation (■). Extracellular pH changes (A) were monitored by pH electrodes (see "Materials and Methods"). The constitutive pH level of the culture medium averages at 5.6 ±0.2. Extracellular [K⁺] changes (B) in the culture medium were determinated by atomic absorption spectroscopy (see "Materials and Methods"). All values represent the means ± standard error of at least three independent experiments each.
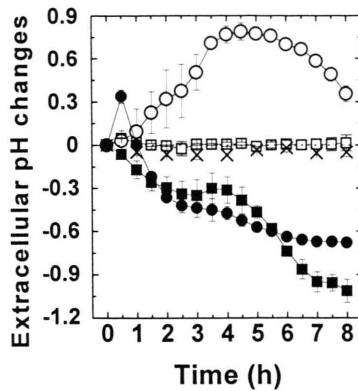
Fig. 4. Long term courses for extracellular pH changes in chickpea cell suspensions in response to elicitation and treatment with different effectors.

At time "zero" distilled water (×), *Pmg* elicitor (50 μg ml^{-1}, ●), cantharidin (10 μM, ○) or staurosporine (1 μM, □) were added separately to the cell suspensions. Further staurosporine (1 μM) was added 10 min prior to *Pmg* elicitation (■). Extracellular pH changes were monitored by pH electrodes (see "Materials and Methods"). The dotted line marks the constitutive pH level of the culture medium, which averages 5.6 ±0.2. Values represent the means ± standard error of three independent experiments each.

of the oxidative burst with DPI (data not shown) whereas solely added DPI had no effect on the constitutive pH level (Fig. 5). Different other inhibitors like the G-protein modulator suramin, the phospholipase C inhibitor neomycin or the anion channel blocker A-9-C inhibited both, the elicitor-induced extracellular alkalinisation as well as the subsequent extracellular acidification (Table I).

Elicitation of transient K$^+$ fluxes

In chickpea cell suspensions application of *Pmg* elicitor increased the extracellular K$^+$ concentration with a maximum of 0.4 μmol g FW^{-1} at about 30 min after elicitor addition which was followed by a decrease of extracellular K$^+$ levels (Fig. 3B). Water-treated cells showed a constant decrease of K$^+$ in the culture medium (Fig. 3B) which nearly led to a complete uptake of K$^+$ ions from the medium after several hours (data not shown). Similar to the elicitor-induced extracellular alkalinisation preincubation of cells with staurosporine strongly inhibited the elicitor-caused K$^+$ efflux whereas staurosporine alone induced a small but transient increase of extracellular K$^+$ (Fig. 3B). An application of cantharidin (10 μM) resulted in a clear release of cellular K$^+$ into the medium with a delay of 30 min (Fig. 3B). In contrast to the data obtained for extracellular alkalinisation preincuba-

preincubation with DPI (5 μM) whereas the following acidification of the medium showed normal extent (Fig. 5A). Also the cantharidin-induced alkalinisation reaction was decreased by inhibition
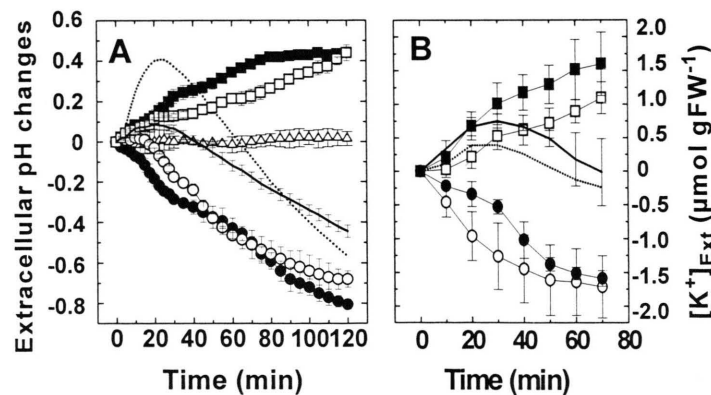
Fig. 5. Effect of DPI, fusicoccin and DCCD on extracellular pH changes (A) and extracellular K$^+$ changes (B) in untreated and *Pmg*-elicited chickpea cell suspensions.

At time "zero" *Pmg* elicitor (50 μg ml^{-1}, dashed line without symbol), DPI (5 μM, △) fusicoccin (5 μM, ○) or DCCD (10 μM, □) were added separately to the cell suspensions. Fusicoccin (5 μM, ●) and DCCD (10 μM, ■) were also added synchronously whereas DPI (5 μM, line without symbol) was applied 10 min prior to treating cells with *Pmg* elicitor (50 μg ml^{-1}). Extracellular pH changes (A) were monitored by pH electrodes (see "Materials and Methods"). The constitutive pH level of the culture medium averaged at 5.6 ±0.2. Extracellular [K$^+$] changes (B) in the culture medium were determinated by atomic absorption spectroscopy (see "Materials and Methods"). All values represent the means ± standard error of at least three independent experiments each.

tion with DPI could not inhibit the subsequent elicitor response and even led to a light increase of the elicitor-induced K^+ efflux (Fig. 5B). Furthermore, sole addition of DPI also induced a small constant increase of extracellular K^+ (data not shown).

Activation of defence related genes

Treatment of cultured chickpea cells with *Pmg* elicitor results in rapid accumulation of transcripts of several defence related genes (Fig. 6). Within the group of PR proteins we measured gene activation as transcript formation for a class III acidic chitinase (PR-8a), a class I basic chitinase (PR-3) and the thaumatin-like protein PR-5b. The other group of genes which represents the phytoalexin response is encoding isoflavone reductase (IFR), a specific enzyme of pterocarpan and medicarpin biosynthesis (Ichinose *et al.*, 2000). The elicitor response depends on protein phosphorylation. Pretreatment of cells with staurosporine (Fig. 6) or the anion channel blocker A-9-C (data not shown)

Fig. 6. Northern analyse of elicitor-induced activation of defence related genes and effects of protein phosphatase and protein kinase inhibitors as well as modulators of plasma membrane H^+-ATPase.
Chickpea cells were treated with *Pmg* elicitor (50 μg ml^{-1}) and cantharidin (25 μM) for two hours and total RNA was extracted. For inhibition studies the following effectors were preincubated 15 min prior to elicitation: staurosporine (2.5 μM), cypermetrin (25 μM), fusicoccin (5 μM) and DCCD (10 μM). 20 μg of total RNA was separated by electrophoresis in formaldehyde-containing 1.0% agarose gels, blotted onto nylon membranes and hybridised with DIG-UTP labelled cRNA probes for isoflavone reductase (*IFR*), basic chitinase (PR-3; *BC*), acidic class III chitinase (PR-8a; *AC*), a thaumatin-like protein (PR-5b; *TLP*) and ubiquitin (*Ubi*).

abolished transcript accumulation of all genes. Cantharidin triggered mRNA accumulation even in the absence of any elicitor (Fig. 6) whereas cypermetrin, an inhibitor of protein phosphatase 2B, had no effect on mRNA accumulation (Levine *et al.*, 1994). Incubation with only staurosporine had also no effect but simultaneous application of cantharidin and staurosporine led to an intermediate response, indicating that a balance of phosphorylated target proteins is necessary for transcriptional up-regulation. The level of polyubiquitin mRNA (Ubi; Fig. 6) was not affected by application of any tested substance, indicating that all treatments caused a specific down- or up-regulation of gene expression.

Influence of modulators of plasma membrane H^+-ATPase on early elicitor responses

In untreated cell suspensions fusicoccin, which activates the PM H^+-ATPase by enhanced binding with 14-3-3 proteins (Baunsgaard *et al.*, 1998) induced a pronounced extracellular acidification and a reduction of external K^+ levels (Fig. 5). In contrast the PM H^+-ATPase inhibitor DCCD (Cid *et al.*, 1987) stimulated a weak but constant alkalinisation and led to a strong efflux of K^+ into the medium (Fig. 5). If separately added, both modulators only weakly induced defence related mRNAs (Fig. 6) and also had no influence on the low constitutive H_2O_2 concentration in the cell suspensions (Fig. 7). When fusicoccin was applied together with *Pmg* elicitor the elicitor-induced alkalinisation was completely inhibited whereas the acidification reaction showed normal extent. The elicitor-stimulated increase of external K^+ was also inhibited and the K^+ flux was reverted to a clear decrease (Fig. 5). The elicited H_2O_2 production was long lastingly extended (Fig. 7). Accumulation of mRNAs was not modified in comparison to elicitor application only but the cantharidin response seemed to be lowered (Fig. 6). Elicitation with simultaneous application of DCCD decreased the elicitor-induced alkalinisation and completely inhibited the subsequent elicitor-induced acidification. The elicited increase of external K^+ was clearly enhanced but the following elicitor induced decrease of K^+ in the medium was inhibited
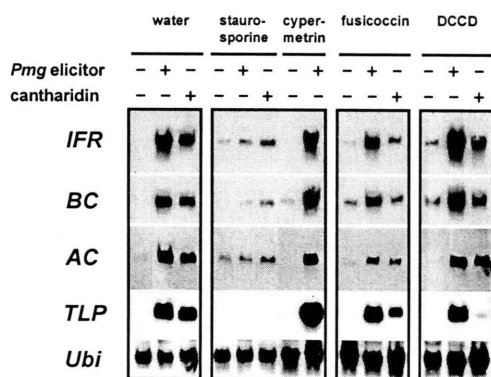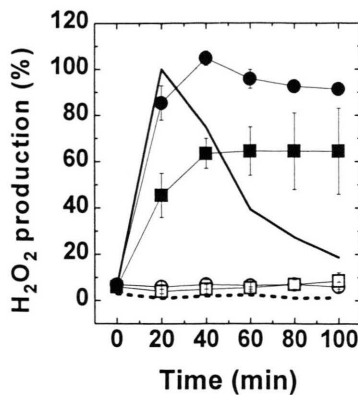
Fig. 7. Effect of DDC, fusicoccin and DCCD on the *Pmg* elicitor stimulated H_2O_2 production in chickpea cell suspensions.

At time "zero" *Pmg* elicitor (50 µg ml^{-1}, line without symbol), FC (5 µM, ○) or DCCD (10 µM, □) were added separately to the cell suspensions. DDC (1 mM, dashed line), FC (5 µM, ●) and DCCD (10 µM, ■) were also added synchronously to the cells with *Pmg* elicitor (50 µg ml^{-1}). Every 20 min aliquots were withdrawn from the cell suspension and the H_2O_2 production was monitored by a luminol chemiluminescence assay (see "Materials and Methods"). The maximum concentration of H_2O_2 which accumulated in response to *Pmg* elicitor treatment was defined as 100%. Values represent the means ±SE of three independent experiments each.

(Fig. 5). The elicited H_2O_2 production was slightly decreased but long lastingly extended (Fig. 7). Similar to the results obtained with fusicoccin regarding activation, inhibition of H$^+$-ATPase activity with DCCD did not suppress elicitor-induced transcript accumulation. Surprisingly, the cantharidin-induced expression of the IFR gene seemed to be sensitive to DCCD (Fig. 6).
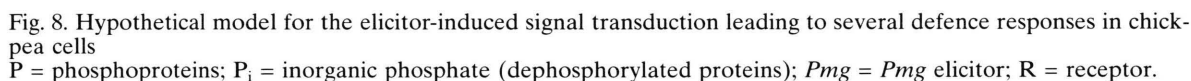
Discussion

Protein phosphorylation in signal transduction

Corresponding with data obtained for other plants (Felix *et al.*, 1993; Viard *et al.*, 1994; Suzuki *et al.*, 1999) protein phosphorylation is an essential component in the elicitor-initiated signal transduction leading to several defence responses in chickpea suspension cells (Fig. 8). The transient character of oxidative burst (Fig. 1), extracellular pH changes (Fig. 3A) and K$^+$ fluxes (Fig. 3B) indicate that activation and inactivation of the early elicitor-induced signal transduction pathway are regu-

lated through a co-ordinated phosphorylation/dephosphorylation cycle as suggested by Otte and Barz (1996). Putative protein kinase activation by elicitor or the inhibition of protein dephosphorylation by cantharidin shifts the constitutive phosphorylation poise of relevant pathway components to their phosphorylated form which leads to signal transduction activation as assumed by Levine *et al.* (1994). Protein kinases associated with defence related gene activation are rapidly induced in other plants (Ligterink *et al.*, 1997; Suzuki *et al.*, 1999) and recently, a protein phosphatase which inactivates MAP kinases has been reported (Gupta *et al.*, 1999).

Further components in signal transduction

In suspension cells of chickpea the strong inhibitory effects of the anion channel blocker A-9-C (Table I) indicate the functional priority and the influences of ion fluxes on the elicitor-induced signal transduction. This corresponds with data received for other plants (Jabs *et al.*, 1997, Rajasekhar *et al.*, 1999). Recently, gain and loss of function experiments utilising pharmacological agents and northern blot analysis revealed that ROS are not a part of the signal transduction chain leading to defence related gene activation (Hein and Barz, unpublished). Contradictory to data obtained for some other plant cell suspensions (Tavernier *et al.*, 1995; Jabs *et al.*, 1997; Zimmermann *et al.*, 1997) our studies on the extracellular Ca^{2+} dependence of the oxidative burst in chickpea cells (Table I) failed to show an effect. In contrast, protoplast suspensions of chickpea cells loose their ability to react with an oxidative burst after elicitation in Ca^{2+} free medium (Pachten and Barz, unpublished). Therefore, we have to evaluate if in chickpea the oxidative burst only needs very low amounts of external Ca^{2+} (Stäb and Ebel, 1987) which might be released out of the cell wall. In chickpea cells the phospholipase C inhibitor neomycin and G-protein modulators act as a potent blocker of the oxidative burst and extracellular pH changes (Table I) which might be due to the putative participation of the phospholipase C mediated inositol phosphate metabolism and intracellular Ca^{2+} stores in elicitor signal transduction (Legendre *et al.*, 1993; Toyoda *et al.*, 1993).

Fig. 8. Hypothetical model for the elicitor-induced signal transduction leading to several defence responses in chickpea cells
P = phosphoproteins; P_i = inorganic phosphate (dephosphorylated proteins); *Pmg* = *Pmg* elicitor; R = receptor.

Oxidative burst

The NADPH oxidase inhibitor DPI (O'Donnell *et al.*, 1993) completely inhibits the elicitor-stimulated H_2O_2 accumulation in suspension cultures of chickpea in a final concentration of 5 μM corresponding with data for complete inhibition in other plant cells (Levine *et al.*, 1994; Desikan *et al.*, 1996; Jabs *et al.*, 1997; Pugin *et al.*, 1997). In chickpea cells the H_2O_2 burst has also been determined as a DPI (5 μM) sensitive O_2 consumption (Otte, 1998). The H_2O_2 burst is preceded by the generation of $\cdot O_2^-$. Using the SOD inhibitor DDC the elicitor-induced H_2O_2 accumulation is completely inhibited (Auh and Murphy, 1995; Desikan *et al.*, 1996). Bolwell *et al.* (1998) recently showed that DDC itself removes H_2O_2 from solution but in chickpea cells the induced $\cdot O_2^-$ production increases simultaneously (Fig. 2). Therefore, we assume a dismutation of $\cdot O_2^-$ to H_2O_2 via extracellular SOD (Desikan *et al.*, 1996). For suspension cells this hypothesis is supported by detection of DDC-sensitive SOD activity in cell wall protein extracts (Otte, 1998). Extracellular SOD has also been found in needles of *Pinus sylvestris* L. (Streller and Wingsle, 1994). Corresponding to our

assumption of $\cdot O_2^-$ dismutation we were able to detect $\cdot O_2^-$ accumulation in chickpea cells, but only in short time experiments (max. 30 min) because sole addition of DDC clearly decreased viability of suspension cells to 75% even after 1 h. This phenomenon rendered later data uncertain and it might be attributed to the accumulation of constitutively produced $\cdot O_2^-$ (Fig. 2). In contrast to the H_2O_2 burst and its corresponding O_2 consumption, the elicitor stimulated $\cdot O_2^-$ production was only inhibited by high concentrations of DPI (20 μM; Fig. 2) as shown for neutrophils (Hampton and Winterbourn, 1995). Due to this data a DPI insensitive elicitor stimulated intracellular $\cdot O_2^-$ production (Allan and Fluhr, 1997) or a negative influence of DDC on the inhibitory DPI effect (Murphy and Auh, 1996) should be considered. Nevertheless, if we take all our data together we assume that in chickpea cells the oxidative burst is driven by a PM bound ROS generating enzyme with the produced $\cdot O_2^-$ being dismutated to H_2O_2 either spontaneously or by putative SOD activity. Further experiments will have to show if the ROS generating enzyme is similar to the phagocytic NADPH oxidase of mammal neutrophils as it has been suggested for many plants (Lamb and Dixon, 1997; Keller *et al.*, 1998).

Elicitation of extracellular alkalinisation and subsequent acidification

As known for the neutrophil NADPH oxidase (Segal, 1995) the assumed catalytic dismutation of $\cdot O_2^-$ to H_2O_2 via the SOD reaction consumes protons in the medium of cell suspensions which should lead to extracellular alkalinisation. We assume that the elicitor-induced alkalinisation in chickpea cultures is substantially (80%) the consequence of this H^+ consumption by the oxidative burst (Fig. 8). Rouet-Mayer *et al.* (1997) also described a 60 percentage inhibition of the medium alkalinisation in tobacco cultures by the DPI analogue diphenyliodonium. Because of H_2O_2 consuming reactions in the cell wall (for example peroxidative insolubilisation of cell wall proteins or other phenolic compounds) it is not possible to determine a stoichiometric relation between H_2O_2 and the elicitor-induced extracellular alkalinisation. In the literature numerous reports on elicitor-stimulated transient alkalinisation in the media of plant cell cultures can be found (Felix *et al.*, 1993; Nürnberger *et al.*, 1994; Jabs *et al.*, 1997; Pugin *et al.*, 1997) but in all these studies a following external acidification reaction as we could show with chickpea cells (Fig. 4) was not described. Therefore, it is evident that this acidification reaction should also be looked for in the other cell culture systems. Vera-Estrella *et al.* (1994) reported a reversible phosphorylation of the PM H^+-ATPase of tomato cells after application of different elicitors. The H^+-ATPase was activated by dephosphorylation whereas the phosphorylated state was inactive. The results of our inhibitor studies with chickpea cell cultures correspond to these findings (Fig. 5). Thus, an activated PM H^+-ATPase is responsible for the acidification in the second phase of extracellular pH changes and might also lead to an increase (20%) of the elicitor-induced alkalinisation when in its inactivated enzyme form (Fig. 8). These pH changes are independent of inhibition of ROS formation (Fig. 5A). On the other hand, the H_2O_2 formation is not influenced by the elicitor-induced extracellular pH changes. Curves of H_2O_2 production in cell suspensions which were buffered to pH 5.7 by 10 mM 2-morpholino-ethanesulfonic acid (data not shown) were identical to the data obtained in non buffered experiment medium as shown in Fig. 1. Interestingly modula-tion of the activation status of the PM H^+-ATPase with fusicoccin or DCCD prevented down-regulation of the oxidative burst (Fig. 7).

$K^/H^+$ exchange response

We suppose that in chickpea cells elicitor-induced H^+ fluxes over the PM cause opposite and simultaneous K^+ fluxes (Fig. 3). Baker *et al.* (1993) described such K^+/H^+- fluxes as an exchange response. This exchange response could be driven by a PM potential difference which is normally built up by H^+ export (Briskin and Gawienowski, 1996). We assume that both elicitor and the protein phosphatase inhibitor cantharidin stabilise the phosphorylated, inactive form of the PM H^+-ATPase. A resulting net H^+ influx supports the extracellular alkalinisation and also stimulates the corresponding K^+ efflux (Fig. 3). This stimulated K^+ flux is insensitive to DPI (Fig. 5) indicating its independence of the postulated H^+ consumption reaction during oxidative burst.

PM H^+-ATPase and defence gene activation

Experiments on loss and gain of function lead us to suggest that activity of the PM H^+-ATPase is not a necessary component for signal transduction leading to defence gene activation in suspension cells of chickpea (Fig. 6). In contrast fusicoccin was reported to induce several genes encoding PR proteins in tomato plants (Roberts and Bowles, 1999; Schaller and Oecking, 1999) and tobacco cells (Fukuda, 1996). In chickpea cells neither pretreatment with fusicoccin nor DCCD was able to abolish elicitor-induced gene activation but fusicoccin, as well as DCCD in the case of TLP gene, seemed to counteract the cantharidin response (Fig. 6). Interestingly, fusicoccin was able to suppress transcript induction of genes encoding systemic wound response proteins in tomato plants (Schaller and Oecking, 1999) and in some cases defence related gene activation in tobacco cells (Fukuda, 1996). Possibly fusicoccin is able to modify the regulation of protein kinase(s) or phosphatase(s) involved in signal transduction by interaction with regulatory 14-3-3 proteins (Aitken, 1996). These data obtained with different plant systems stress the complexity of regulatory sys-

tems for transduction of elicitor signals. Different solutions obviously are operating. We summarise our results in the working hypothesis depicted in Fig. 8.

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