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

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Cicer arietinum, Defence Related Gene Activation, Extracellular Acidification, Oxidative Burst

Elicitation of cultured chickpea (Cicer arietinum L.) cells stimulates a signal transduction pathway leading to several rapid responses: (1) oxidative burst, (2) extracellular alkalisation, (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 soflavone reductase. All these elicitor-induced responses are inhibited by the Ser/Thr protein kinase inhibitor staurosporine and the anion channel blocker N,N’-di-carboxylic acid; BC, basic chitinase; DCCD, N,N’-di-cyclohexylcarbodiimide; DPI, diphenylene iodonium; IFR, isoflavone reductase; PM, plasma membrane; TLP, thaumatin-like protein; Ubi, ubiquitin.

Abbreviations: AC, acidic chitinase; A-9-C, anthracene-9-carboxylic acid; BC, basic chitinase; DCCD, N,N’-di-cyclohexylcarbodiimide; DDC, diethylildithiocarbamate; DPI, diphenylene iodonium; IFR, isoflavone reductase; PM, plasma membrane; Pmg, Phytophthora megasperma f. sp. glycinea (new term P. sojae); TLP, thaumatin-like protein; Ubi, ubiquitin.

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 (O2•−), hydroxyl radical (OH) and hydrogen peroxide (H2O2). Oxidative burst is often accompanied by other early defence responses: (1) peroxidative insolubilisation of plant cell wall proteins (Otto 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 pathway leading to several rapid responses: (1) oxidative burst, (2) extracellular alkalisation, (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 soflavone reductase. All these elicitor-induced responses are inhibited by the Ser/Thr protein kinase inhibitor staurosporine and the anion channel blocker N,N’-di-carboxylic acid; BC, basic chitinase; DCCD, N,N’-di-cyclohexylcarbodiimide; DPI, diphenylene iodonium; IFR, isoflavone reductase; PM, plasma membrane; TLP, thaumatin-like protein; Ubi, ubiquitin.

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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$_2$O$_2$ (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$_2$O$_2$ generation

H$_2$O$_2$ 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×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 micro-electrode (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 (Chomzynski 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 20x SSPE and hybridized over night at high stringency in 5x SSC, 1% N-lauroyl sarcosine, 0.02% SDS, 2% blocking reagent (Boehringer, Germany) and 50% formamide at 68 °C. Digoxigenin-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 0.1x SSC, 0.1x 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 H₂O₂ 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 H₂O₂ (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 H₂O₂ 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; IC₅₀: 0.2 µM) whereas the protein phosphatase inhibitor cantharidin accelerated, potentiated and extended the elicited H₂O₂ release (Otte and Barz, 1996). With a delay of 30 min cantharidin (10 µM; EC₅₀ 7.5 µM) even stimulated a several hours-lasting H₂O₂ burst in

Fig. 1. Short (A) and long term (B) courses of H₂O₂ production in chickpea cell suspensions in response to elicitation and treatment with different effectors. At time “zero” distilled water (x), 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 H₂O₂ concentration was determined by a luminol chemiluminescence assay (see “Materials and Methods”). B: The maximum concentration of H₂O₂ 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) preincubation 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 (Pineros 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.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Function</th>
<th>Inhibition of oxidative burst</th>
<th>Inhibition of extracellular alkalinisation</th>
<th>Inhibition of extracellular acidification</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPI (5 μM)</td>
<td>NADPH oxidase inhibitor</td>
<td>100% (IC$_{50}$: 0.25 μM)</td>
<td>80%</td>
<td>15%</td>
</tr>
<tr>
<td>A-9-C (100 μM)</td>
<td>Cl$^-$ channel blocker</td>
<td>85% (IC$_{50}$: 20 μM)</td>
<td>75%</td>
<td>100%</td>
</tr>
<tr>
<td>Niflumene acid (200 μM)</td>
<td>Cl$^-$ channel blocker</td>
<td>85% (IC$_{50}$: 90 μM)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Neomycin (200 μM)</td>
<td>Phospholipase C inhibitor</td>
<td>85% (IC$_{50}$: 115 μM)</td>
<td>50%</td>
<td>100%</td>
</tr>
<tr>
<td>Suramin (100 μM)</td>
<td>G-protein modulator</td>
<td>45% (IC$_{50}$: 110 μM)</td>
<td>70%</td>
<td>75%</td>
</tr>
<tr>
<td>Mastoparan (20 μM)</td>
<td>G-protein modulator</td>
<td>90% (IC$_{50}$: 10 μM)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>EGTA (3 mM)</td>
<td>Ca$^{2+}$ chelator</td>
<td>0%</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Verapamil (200 μM)</td>
<td>Ca$^{2+}$ channel inhibitor</td>
<td>10%</td>
<td>30%</td>
<td>0%</td>
</tr>
</tbody>
</table>
Fig. 2. Time course for \( \mathrm{O}_2^- \) accumulation generated by chickpea cells. At time “zero” DDC (1 mM) was added together either with distilled water (○) or \( \text{Pmg} \) elicitor (50 µg ml\(^{-1}\), ●) to the cell suspensions. Further DDC (1 mM) and \( \text{Pmg} \) 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 \( \mathrm{O}_2^- \) 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 \( \text{Pmg} \) 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 (EC\(_{50}\) 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. 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.

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 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-

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 determined 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) 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 alkalisation 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 H₂O₂ concentration in the cell suspensions (Fig. 7). When fusicoccin was applied together with Pmg elicitor the elicitor-induced alkalisation 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 H₂O₂ production was long lastingly extended (Fig. 6). 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 alkalisation 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$_2$O$_2$ 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$_2$O$_2$ production was monitored by a luminol chemiluminescence assay (see “Materials and Methods”). The maximum concentration of H$_2$O$_2$ 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$_2$O$_2$ production was slightly decreased but long lasting 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 regulated 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 (Ligerink *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, Raja- sekhar *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; Zimmernmann *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).
Oxidative burst

The NADPH oxidase inhibitor DPI (O’Donnell et al., 1993) completely inhibits the elicitor-stimulated H₂O₂ 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 H₂O₂ burst has also been determined as a DPI (5 μM) sensitive O₂⁻ consumption (Otte, 1998). The H₂O₂ burst is preceded by the generation of O₂⁻. Using the SOD inhibitor DDC the elicitor-induced H₂O₂ accumulation is completely inhibited (Auh and Murphy, 1995; Desikan et al., 1996). Bolwell et al. (1998) recently showed that DDC itself removes H₂O₂ from solution but in chickpea cells the induced O₂⁻ production increases simultaneously (Fig. 2). Therefore, we assume a dismutation of O₂⁻ to H₂O₂ 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 O₂⁻ dismutation we were able to detect O₂⁻ 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 O₂⁻ (Fig. 2). In contrast to the H₂O₂ burst and its corresponding O₂ consumption, the elicitor stimulated O₂⁻ 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 O₂⁻ 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 O₂⁻ being dismutated to H₂O₂ 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 \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \) 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 \( \text{H}^+ \) 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 \( \text{H}_2\text{O}_2 \) consuming reactions in the cell wall (for example per-oxidative insolubilisation of cell wall proteins or other phenolic compounds) it is not possible to determine a stoichiometric relation between \( \text{H}_2\text{O}_2 \) 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; Nürnberg 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 \( \text{H}^+\text{-ATPase} \) of tomato cells after application of different elicitors. The \( \text{H}^+\text{-ATPase} \) 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 \( \text{H}^+\text{-ATPase} \) 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 \( \text{H}_2\text{O}_2 \) formation is not influenced by the elicitor-induced extracellular pH changes. Curves of \( \text{H}_2\text{O}_2 \) 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 modulation of the activation status of the PM \( \text{H}^+\text{-ATPase} \) with fusicoccin or DCCD prevented down-regulation of the oxidative burst (Fig. 7).

\( \text{K}^+\text{/H}^+ \) exchange response

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

PM \( \text{H}^+\text{-ATPase} \) and defence gene activation

Experiments on loss and gain of function lead us to suggest that activity of the PM \( \text{H}^+\text{-ATPase} \) 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 pre-treatment 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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