Receptor-Mediated Increase in Cytoplasmic Free Calcium Required for Activation of Pathogen Defense in Parsley

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Transient influx of Ca\(^{2+}\) constitutes an early element of signaling cascades triggering pathogen defense responses in plant cells. Treatment with the Phytophthora sojae-derived oligopeptide elicitor, Pep-13, of parsley cells stably expressing apoaequorin revealed a rapid increase in cytoplasmic free calcium ([Ca\(^{2+}\)]\(_{cyt}\)) which peaked at \(-1\) \(\mu\)M and subsequently declined to sustained values of 300 nM. Activation of this biphasic [Ca\(^{2+}\)]\(_{cyt}\) signature was achieved by elicitor concentrations sufficient to stimulate Ca\(^{2+}\) influx across the plasma membrane, oxidative burst, and phytoalexin production. Sustained concentrations of [Ca\(^{2+}\)]\(_{cyt}\) but not the rapidly induced [Ca\(^{2+}\)]\(_{cyt}\) transient peak are required for activation of defense-associated responses. Modulation by pharmacological effectors of Ca\(^{2+}\) influx across the plasma membrane or of Ca\(^{2+}\) release from internal stores suggests that the elicitor-induced sustained increase of [Ca\(^{2+}\)]\(_{cyt}\) predominantly results from the influx of extracellular Ca\(^{2+}\). Identical structural features of Pep-13 were found to be essential for receptor binding, increases in [Ca\(^{2+}\)]\(_{cyt}\), and activation of defense-associated responses. Thus, a receptor-mediated increase in [Ca\(^{2+}\)]\(_{cyt}\) is causally involved in signaling the activation of pathogen defense in parsley.

INTRODUCTION

Cytoplasmic free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{cyt}\)) serves as a second messenger in plant processes as diverse as root nodule formation, phytochrome phototransduction, stomatal closure, geotropism, circadian rhythm, pollen tube growth, and stress adaptation (Rudd and Franklin-Tong, 1999). Stimulus-specific and spatially and temporally defined Ca\(^{2+}\) signatures of characteristic magnitude, frequency, and duration are assumed to maintain signal specificity of transduction cascades (Thuleau et al., 1998; Trewavas, 1999). Subsequently, the binding of [Ca\(^{2+}\)]\(_{cyt}\) to calmodulin, Ca\(^{2+}\)-dependent protein kinases, Ca\(^{2+}\)-dependent protein phosphatases, Ca\(^{2+}\)-gated ion channels, or Ca\(^{2+}\)-activated phospholipases facilitates downstream signal transduction directed toward activation of a signal-specific cellular response (Blumwald et al., 1998).

Expression of the Aequorea aequorea apoaequorin gene in the cytoplasm of plant cells provides a means for accurate, noninvasive quantification of changes in [Ca\(^{2+}\)]\(_{cyt}\) (Knight et al., 1991). When reconstituted with coelenterazine, holoequorin acts as a bioluminescent indicator of [Ca\(^{2+}\)]\(_{cyt}\). Since the pioneering work of Knight et al. (1991), aequorin technology has been widely applied in plants to report changes in [Ca\(^{2+}\)]\(_{cyt}\) in response to abiotic stimuli, such as touch, wind, cold, heat, and drought (Knight et al., 1991, 1992, 1996, 1997; Haley et al., 1995; Gong et al., 1998; Plieth et al., 1999); blue light (Lewis et al., 1997); circadian rhythm (Johnson et al., 1995); ozone (Clayton et al., 1999); anoxia (Sedbrook et al., 1996); oxidative stress (Price et al., 1994); and hypoosmotic shock (Chandra and Low, 1997; Takahashi et al., 1997; Cessna et al., 1998).

Numerous recent studies have provided evidence that Ca\(^{2+}\) plays a pivotal role in activating the plant’s surveillance system against attempted microbial invasion (Yang et al., 1997; Scheel, 1998). Activation of plant defense is believed to be receptor mediated through recognition of pathogen-derived elicitors (Yang et al., 1997; Scheel, 1998; Nürnberger, 1999). In contrast to elicitors from phytopathogenic bacteria, elicitors of fungal or oomycete origin appear to be recognized by high-affinity receptors residing in the plasma membrane of plant cells. Although several such plasma membrane binding sites have been characterized kinetically and structurally, our knowledge of the molecular mode of fungal pathogen perception in plants remains fragmentary: only one elicitor receptor has been isolated thus far, a soybean 70-kD plasma membrane protein that binds Phytophthora sojae-derived β-glucans (Umemoto et al., 1997).

Receptor–ligand interaction initiates an intracellular signal transduction cascade that mediates activation of the defense against the pathogen. Cellular components shown to be modulated by elicitor treatment include GTP binding proteins (Bischoff et al., 1999); plasma membrane ion channels...
(Thuleau et al., 1998); reactive oxygen intermediates (Lamb and Dixon, 1997); nitric oxide (Delledonne et al., 1998); lipid-derived metabolites (Chandra et al., 1997); and alterations in the phosphorylation status of various proteins catalyzed by serine/threonine protein kinases, protein phosphatases, and post-translationally activated mitogen-activated protein kinase (MAP kinase) cascades (Sopory and Munshi, 1998). Remarkably, many of these elements thought to be implicated in elicitor signaling have been identified in various plants, suggesting evolutionary conservation of signaling modules in plant pathogen defense (Yang et al., 1997; Scheel, 1998).

Plasma membrane ion channels are rapidly activated by pathogen infection or elicitor treatment of plant cells. In particular, extracellular alkalization, Ca\(^{2+}\) influx, and effluxes of K\(^+\) and Cl\(^{-}\) lead to depolarization of the plasma membrane (Scheel, 1998). Extracellular Ca\(^{2+}\) appears to be crucial to induction of plant defense against pathogens (Yang et al., 1997; Scheel, 1998). Elicitor-responsive Ca\(^{2+}\)-permeable ion channels residing in the plasma membrane of plant cells may mediate elicitor-induced Ca\(^{2+}\) influx and subsequently lead to increased amounts of [Ca\(^{2+}\)]\(_{cyt}\) (Gelli et al., 1997; Zimmermann et al., 1997).

Rapidly induced transient increase of [Ca\(^{2+}\)]\(_{cyt}\) was previously monitored in apoaequorin-transformed tobacco cells that had been treated with crude elicitors derived from yeast or Gliocladium deliquescentes (Knight et al., 1991). In contrast, harpin from Erwinia amylovora, which induces an oxidative burst, defense-related gene expression, and hypersensitive cell death in tobacco, did not affect [Ca\(^{2+}\)]\(_{cyt}\) in this plant (Chandra et al., 1997). Only recently were defined fungal chitin fragments or oomycete-derived glucan fragments shown to increase [Ca\(^{2+}\)]\(_{cyt}\) in soybean cells (Mithöfer et al., 1999). Crucial questions, however, as to whether changes in [Ca\(^{2+}\)]\(_{cyt}\) are receptor mediated and thus involved in signal activation of defense responses to pathogens remain to be resolved.

The nonhost resistance response of parsley (Petroselinum crispum) leaves to infection with zoospores of the phytopathogenic oomycete P. sojae was found to be closely mimicked in parsley cell cultures treated with cell wall-derived elicitors (Hahlbrock et al., 1995). An oligopeptide fragment (Pep-13) of a 42-kD P. sojae cell wall glycoprotein stimulates transcriptional activation of defense-related genes and phytoalexin production (Nürnberger et al., 1994). The binding of Pep-13 to a 100-kD parsley plasma membrane receptor (Nürnberger et al., 1995; Nennstiel et al., 1998) rapidly stimulates Ca\(^{2+}\) influx, effluxes of K\(^+\) and Cl\(^{-}\), extracellular alkalization, production of reactive oxygen species (ROS), and post-translational activation of a MAP kinase (Nürnberger et al., 1994; Jabs et al., 1997; Ligterink et al., 1997). Extracellular Ca\(^{2+}\) was found to be indispensable for activation of all these plant responses (Nürnberger et al., 1994; Ligterink et al., 1997). Use of peptides structurally related to Pep-13 revealed a functional link between elicitor perception and activation of Ca\(^{2+}\) influx as a requirement for subsequent production of superoxide anions. ROS themselves are both necessary and sufficient to trigger pathogen defense responses in parsley (Nürnberger et al., 1994; Jabs et al., 1997). Patch-clamp analyses demonstrated the receptor-mediated activation of a Pep-13-responsive Ca\(^{2+}\)-permeable plasma membrane ion channel (Zimmermann et al., 1997). We now show that parsley cells stably expressing the apoaequorin gene respond to elicitor treatment with a characteristic biphasic [Ca\(^{2+}\)]\(_{cyt}\) signature consisting of a rapidly induced [Ca\(^{2+}\)]\(_{cyt}\) transient peak followed by sustained concentrations of [Ca\(^{2+}\)]\(_{cyt}\). Intriguingly, although both phases of the Pep-13-induced [Ca\(^{2+}\)]\(_{cyt}\) response in parsley cells are due to Pep-13 receptor activation, only sustained increases in [Ca\(^{2+}\)]\(_{cyt}\) constitute an element of the signaling cascade triggering pathogen defense in parsley.

RESULTS

Establishment of Parsley Cell Lines Stably Expressing Cytoplasmic Apoaequorin

Particle bombardment technology was used to introduce a DNA cassette encoding apoaequorin into suspension-cultured parsley cells (Finer et al., 1992; Frohnmeyer et al., 1999). When holoaequorin was reconstituted with coelenterazine, 27 of 37 hygromycin-resistant calli exhibited Ca\(^{2+}\)-dependent bioluminescence. Cell suspensions were established from those calli showing the greatest amounts of reconstituted bioluminescent aequorin (Table 1).

DNA gel blot analysis confirmed integration of one to six copies of the apoaequorin-encoding DNA into the parsley genome. Maintenance of the Ca\(^{2+}\)-dependent bioluminescence in parsley cell lines cultivated for >12 months in the absence of selecting antibiotic proved that the integration of the transgene was stable. Phenotypic appearance and growth behavior of transgenic parsley cell lines were indistinguishable from those of nontransformed cell lines. Most importantly, when treated with the P. sojae-derived oligopeptide elicitor Pep-13, apoaequorin-transgenic parsley cell lines produced ROS and furanocoumarin phytoalexins in quantities comparable to those produced by nontransformed cell lines (Table 1). Nevertheless, to avoid misinterpretation of data in consideration of adverse effects of transgene integration on cellular responsiveness, all bioluminometric experiments were routinely performed in triplicate with at least two transgenic cell lines.

Because of the double logarithmic relationship between aequorin bioluminescence and [Ca\(^{2+}\)] (Blinks et al., 1978), relative changes in light emission do not precisely reflect the extent of any change in actual [Ca\(^{2+}\)]. Moreover, because aequorin is consumed during the experiment and because the amount of reconstituted aequorin varies between individual experiments, meaningful analysis of recorded relative light units (RLUs) required conversion into [Ca\(^{2+}\)] by using a
calibration curve established in vitro. All relative changes in bioluminescence monitored were therefore transformed into absolute values for \([\text{Ca}^{2+}]_{\text{cyt}}\).

### Increased \([\text{Ca}^{2+}]_{\text{cyt}}\) in Elicitor-Treated Parsley Cells

In transgenic parsley lines reconstituted aequorin reported a basal \([\text{Ca}^{2+}]_{\text{cyt}}\) level of 40 to 110 nM. Treating the parsley cells with the oligopeptide elicitor Pep-13 produced characteristic changes in \([\text{Ca}^{2+}]_{\text{cyt}}\) (Figure 1), which were similar in all transgenic cell lines listed in Table 1. After a delay of 30 to 40 sec, \([\text{Ca}^{2+}]_{\text{cyt}}\) increased rapidly, peaked at \(\sim 1 \mu \text{M}\) after 2 min, and subsequently decreased to a slowly declining plateau of \(\sim 300 \text{nM}\) during the next 10 to 40 min (Figure 1A). This biphasic response differed greatly from the immediate, transient, small peak detectable after addition of water or organic solvents. In contrast to the peak maximum (phase 1), which varied between 600 and 1100 nM among individual experiments performed with independent transgenic lines, the magnitude of the plateau value (phase 2) and the kinetics of the total response were consistently found to be identical across all lines.

The extent of the \([\text{Ca}^{2+}]_{\text{cyt}}\) response depended on the concentration of elicitor used, becoming saturated at Pep-13 concentrations \(> 5 \text{nM}\) (Figure 1B). Decreasing the elicitor concentration preferentially reduced the transient \([\text{Ca}^{2+}]_{\text{cyt}}\) peak, whereas sustained increases of \([\text{Ca}^{2+}]_{\text{cyt}}\) could be elicited at Pep-13 concentrations as low as 0.25 nM. Lowering the Pep-13 concentration further also diminished the activation of the second phase of the \([\text{Ca}^{2+}]_{\text{cyt}}\) response. Importantly, we found a close quantitative correlation between the elicitor concentrations required to efficiently stimulate increased \([\text{Ca}^{2+}]_{\text{cyt}}\) and phytoalexin formation (Figure 1B). Maximum phytoalexin production was observed only at Pep-13 concentrations that elicited both phases of the \([\text{Ca}^{2+}]_{\text{cyt}}\) response. However, large amounts of phytoalexins (80% of maximum) were also produced in response to Pep-13 concentrations that stimulated only phase 2 of the \([\text{Ca}^{2+}]_{\text{cyt}}\) response. The EC50 value for Pep-13 to induce the sustained \([\text{Ca}^{2+}]_{\text{cyt}}\) plateau (0.2 nM) closely corresponded to the elicitor concentrations required to half-maximally elicit \([\text{Ca}^{2+}]_{\text{cyt}}\) signature observed in luminometric experiments (Table 2; Nürnberger et al., 1994).

In contrast to luminometric analyses of large cell populations (5 \(\times\) 10^3 cells per experiment), in vivo imaging of aequorin activity using photon-counting video equipment permitted analysis of small cell clusters (10 to 20 cells). These experiments were performed to investigate whether the elicitor-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) signature might be the result of cumulative light emission from cell populations responding asynchronously to elicitor with differing lag phases, and whether the \([\text{Ca}^{2+}]_{\text{cyt}}\) plateau might be brought about by alternately oscillating \([\text{Ca}^{2+}]_{\text{cyt}}\) spikes. As shown in Figure 2, the parsley cell clusters responded rather synchronously to elicitor treatment. Maximum light emission was observed between 60 and 160 sec after addition of elicitor (Figure 2A), corresponding precisely to the time of the large \([\text{Ca}^{2+}]_{\text{cyt}}\) spike seen in luminometric assays (Figure 1A). Subsequently, light emission declined (Figure 2B) but remained constantly higher than that observed in untreated cells (not shown) or in cells immediately after the administration of elicitor (first image, Figure 2A). This is reminiscent of the plateau phase monitored in luminometric measurements (Figure 1A). In all experiments performed, the majority of cell clusters responded to elicitor treatment (Figures 2C and 2D). Because the light emission of elicitor-treated parsley cells was synchronous and continuous rather than asynchronous or oscillating, the \([\text{Ca}^{2+}]_{\text{cyt}}\) signature observed in luminometric

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**Table 1. Characterization of Transgenic Parsley Cell Lines Expressing Apoaequorin**

<table>
<thead>
<tr>
<th>Line</th>
<th>Number of Transgene Insertions</th>
<th>Bioluminescence of Reconstituted Aequorin (RLU/sec/mg FW)</th>
<th>Oxidative Burst (Percentage of Untransformed Cells)</th>
<th>Phytoalexin Production (Percentage of Untransformed Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/10</td>
<td>1</td>
<td>1.87 (\times) 10^6</td>
<td>146 (\times) 2</td>
<td>68 (\times) 12</td>
</tr>
<tr>
<td>1/14</td>
<td>4–6</td>
<td>1.98 (\times) 10^6</td>
<td>127 (\times) 12</td>
<td>54 (\times) 29</td>
</tr>
<tr>
<td>1/17</td>
<td>1</td>
<td>2.06 (\times) 10^6</td>
<td>ND^a</td>
<td>40 (\times) 17</td>
</tr>
<tr>
<td>1/18</td>
<td>1</td>
<td>1.41 (\times) 10^6</td>
<td>125 (\times) 10</td>
<td>61 (\times) 28</td>
</tr>
<tr>
<td>1/19</td>
<td>3</td>
<td>1.27 (\times) 10^5</td>
<td>72 (\times) 27</td>
<td>57 (\times) 32</td>
</tr>
<tr>
<td>2/2</td>
<td>2</td>
<td>2.66 (\times) 10^5</td>
<td>95 (\times) 32</td>
<td>112 (\times) 9</td>
</tr>
<tr>
<td>2/5</td>
<td>ND^a</td>
<td>0.33 (\times) 10^5</td>
<td>219 (\times) 20</td>
<td>69 (\times) 17</td>
</tr>
<tr>
<td>2/12</td>
<td>2</td>
<td>6.25 (\times) 10^5</td>
<td>114 (\times) 7</td>
<td>78 (\times) 26</td>
</tr>
</tbody>
</table>

^a Integration of the aequorin construct was investigated by genomic DNA gel blot analysis.

^b Determined by complete discharge of aequorin with excess Ca^{2+}.

^c Oxidative burst measurements were performed in triplicate.

^d Phytoalexin production was quantified whenever cell lines were used for bioluminescence experiments.

^e Not determined.

FW, fresh weight; RLU, relative light units.
Elicitor Specificity of the [Ca$^{2+}$]$_{cyt}$ Response

The 42-kD P. sojae cell wall glycoprotein harboring Pep-13 was tested for its ability to elicit the [Ca$^{2+}$]$_{cyt}$ response in parsley cells. Somewhat unexpectedly, this glycoprotein elicitor did not trigger the large [Ca$^{2+}$]$_{cyt}$ transient peak in the way Pep-13 did. Instead, a lag phase of ~4 min preceded the increase in [Ca$^{2+}$]$_{cyt}$, which reached a sustained value (phase 2) of 300 nM, the same as that of the Pep-13-treated cells, 12 to 15 min after the glycoprotein was added (Figure 3A). Because this elicitor stimulated phytoalexin production in parsley cells as efficiently as Pep-13 did, the characteristic transient [Ca$^{2+}$]$_{cyt}$ spike triggered by Pep-13 appears to be dispensable for activation of pathogen defense-associated responses in parsley.

The impact on parsley [Ca$^{2+}$]$_{cyt}$ of a series of plant defense elicitors was investigated. Interestingly, elicitors that induced phytoalexin production in parsley cells stimulated only phase 2 of the [Ca$^{2+}$]$_{cyt}$ response (Figure 3A). At concentrations sufficient to activate phytoalexin production in parsley cells, harpin, the product of the HRPZ gene from Pseudomonas syringae pv phaseolicola, yielded increased [Ca$^{2+}$]$_{cyt}$ with kinetics and magnitude (~250 nM) comparable to those of the P. sojae glycoprotein elicitor. Furthermore, the polypeptide antibiotic amphotericin B, previously shown to trigger sustained ion fluxes across the plasma membrane as well as phytoalexin production in parsley cells in the absence of elicitor (Jabs et al., 1997), increased the [Ca$^{2+}$]$_{cyt}$ with kinetics and magnitude (~220 nM) very similar to those shown by harpin and the P. sojae glycoprotein elicitor.

In contrast, elicitors incapable of inducing phytoalexin production in parsley cells were either ineffective with respect to increasing [Ca$^{2+}$]$_{cyt}$ (Phytophthora megasperma β-elicitin, β-megaspermin) or induced only a rapid, transient increase in [Ca$^{2+}$]$_{cyt}$ even at concentrations 10-fold greater than those reported from other plant systems to be sufficient to trigger pathogen defense reactions (Figure 3B). The [Ca$^{2+}$]$_{cyt}$ signature induced by fungal N-acetylchitoheptaose was reminiscent of phase 1 of the Pep-13-induced [Ca$^{2+}$]$_{cyt}$ response. Using less-polymerized chitooligosaccharides resulted in concomitant reduction (chitopentaose, not shown) or complete loss (chitotriose, not shown) of their [Ca$^{2+}$]$_{cyt}$-increasing activity. A 15-mer peptide derived from a conserved domain of bacterial flagellin, which was shown to elicit plant defense-associated responses in various dicot plant cells (Felix et al., 1999), increased [Ca$^{2+}$]$_{cyt}$ in parsley cells transiently up to 350 nM.

Among other abiotic stimuli, cold shock reportedly triggers an increase in [Ca$^{2+}$]$_{cyt}$ in apoaequorin-transformed tobacco plants and cultured cells (Knight et al., 1991, 1996; Chandra and Low, 1997). As shown in Figure 3C, addition of ice-cold medium to transgenic parsley cells precipitously induced a [Ca$^{2+}$]$_{cyt}$ spike, which reached a maximum after 20 sec at concentrations >3 μM, subsequently declining to basal values within 150 sec. However, this cold shock could neither increase the [Ca$^{2+}$]$_{cyt}$ to sustained values nor stimulate phytoalexin production in parsley cells.

Requirement of Extracellular Ca$^{2+}$ for Elicitor-Induced Increase of [Ca$^{2+}$]$_{cyt}$

Previous experiments proved that extracellular Ca$^{2+}$ is essential for activation of the multifaceted defense response in elicitor-treated parsley cells (Nürnberger et al., 1994; Jabs et al., 1997; Ligterink et al., 1997). In addition, Pep-13 was
shown to initiate influx of $^{45}\text{Ca}^{2+}$ into parsley cells and to activate a plasma membrane $\text{Ca}^{2+}$-permeable ion channel (Nürnberger et al., 1994; Zimmermann et al., 1997). Thus, an elicitor-induced increase in $[\text{Ca}^{2+}]_{cyt}$ may be a direct consequence of $\text{Ca}^{2+}$ influx through plasma membrane ion channels. Alternatively, generation of inositol trisphosphate (IP$_3$) or cyclic ADP-ribose and activation of their corresponding receptors could mobilize $\text{Ca}^{2+}$ from internal stores, such as the vacuole. As shown in Figure 4A, chelation of extracellular $\text{Ca}^{2+}$ by the chelating agent 1,2-bis(aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) abolished both phases of the Pep-13-induced increase in $[\text{Ca}^{2+}]_{cyt}$. Similarly, EGTA inhibited the $[\text{Ca}^{2+}]_{cyt}$ response in elicitor-treated parsley cells (not shown). Addition of BAPTA to parsley cells after onset of elicitor treatment immediately abrogated both the transient $[\text{Ca}^{2+}]_{cyt}$ peak and the plateau phase of the $[\text{Ca}^{2+}]_{cyt}$ response. In contrast, cold shock, a stimulus known to trigger $\text{Ca}^{2+}$ release from vacuolar stores in addition to influx of extracellular $\text{Ca}^{2+}$ (Knight et al., 1996), evoked a $[\text{Ca}^{2+}]_{cyt}$ transient peak in parsley cells in the absence of BAPTA (Figure 4A). Addition of excess $\text{Ca}^{2+}$ to parsley cells preincubated with BAPTA fully restored the $[\text{Ca}^{2+}]_{cyt}$ response (Figure 4B) and the ability of elicitor-treated parsley cells to produce ROS and phytoalexins (not shown). Thus, irreversible damage of parsley plasma membranes caused by BAPTA appears unlikely. Moreover, the Pep-13-induced $[\text{Ca}^{2+}]_{cyt}$ response of parsley cells treated simultaneously with BAPTA and excess $\text{CaCl}_2$ was indistinguishable from that of parsley cells treated with Pep-13 alone. Modulation of the extracellular free $\text{Ca}^{2+}$ concentration by BAPTA resulted in a concomitant decrease in the ability of elicited parsley cells to mount a $[\text{Ca}^{2+}]_{cyt}$ response and an oxidative burst (Figure 4C). This suggests that induction of both cellular responses is similarly dependent on external $\text{Ca}^{2+}$. Strikingly, as long as the extracellular free $[\text{Ca}^{2+}]$ exceeded the $[\text{Ca}^{2+}]_{cyt}$ parsley cells remained responsive to Pep-13.

Lanthanides ($\text{Gd}^{3+}$, $\text{La}^{3+}$) are frequently used to inhibit $\text{Ca}^{2+}$ importation across the plant plasma membrane. When added to parsley cells either before or together with Pep-13, 1 mM Gd$^{3+}$ abolished the Pep-13-induced $[\text{Ca}^{2+}]_{cyt}$ signature (Figure 4D) and reduced phytoalexin production in elicitor-treated parsley cells by 91%. Use of 1 mM La$^{3+}$ gave similar results (not shown). However, lanthanides are reported both to enter cells at millimolar external concentrations (Shimizu et al., 1997) and to affect plasma membrane $\text{Ca}^{2+}$-ATPase (Quiguamphoix et al., 1990) and $\text{K}^+$ channels (Lewis and Spalding, 1998). Thus, our findings lend further support to the prime role of extracellular $\text{Ca}^{2+}$ for activating plant defense in parsley but do not provide evidence for specific plasma membrane $\text{Ca}^{2+}$ channels to mediate an elicitor-induced $\text{Ca}^{2+}$ influx.

In a series of previous experiments, verapamil, nifedipine, and flunarizine, nonpermeable inhibitors of $\text{Ca}^{2+}$ channels, failed to inhibit elicitor-induced alkalization of the extracellular medium, $\text{Ca}^{2+}$ influx, $\text{K}^+$ and $\text{Cl}^-$ effluxes, oxidative burst, and phytoalexin production in parsley cells (Jabs et al., 1997). These compounds were now found to be also incapable of blocking the elicitor-induced increase in $[\text{Ca}^{2+}]_{cyt}$ after 15 min of preincubation with inhibitor (not shown).

Ruthenium Red (RR), a membrane-permeable $\text{Ca}^{2+}$ channel blocker that predominantly inhibits $\text{Ca}^{2+}$ release from intracellular compartments, was used to elucidate the contribution of internal $\text{Ca}^{2+}$ stores to the elicitor-induced $[\text{Ca}^{2+}]_{cyt}$ signature response. At 100 $\mu$M, RR markedly inhibited

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**Table 2. Correlation between Elicitor-Specific Responses in Parsley Challenged with Pep-13 and Structural Analogs**

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Competitor Activity</th>
<th>$\Delta [\text{Ca}^{2+}]_{cyt}$ (Aequorin)</th>
<th>$\text{H}_2\text{O}_2$ Formation</th>
<th>Phytoalexin Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pep-13</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>VWNQPRGFKVYE</td>
<td>1</td>
<td>1</td>
<td>(0.2 nM)$^b$</td>
<td>(0.19 nM)</td>
</tr>
<tr>
<td>Pep-13/A12</td>
<td>0.7</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>VWNQPRGFKVYAE$^c$</td>
<td>1.3</td>
<td>1.7</td>
<td>1.6</td>
<td>1.3</td>
</tr>
<tr>
<td>Pep-13/A2</td>
<td>1,500</td>
<td>390</td>
<td>280</td>
<td>70</td>
</tr>
<tr>
<td>VANQPRGFKVYE</td>
<td></td>
<td></td>
<td>230</td>
<td></td>
</tr>
<tr>
<td>Pep-10</td>
<td>7,000</td>
<td>—</td>
<td>15,500</td>
<td>3,400</td>
</tr>
<tr>
<td>NQPVRGFKVY</td>
<td></td>
<td></td>
<td>1,100</td>
<td></td>
</tr>
</tbody>
</table>

$^a$The activity index (Pep-X/Pep-13) represents the quotient of the half-maximal concentration of the respective Pep-13 structural derivative (Pep-X) required to stimulate the particular plant response and the half-maximal effector concentration of Pep-13. The difference in $p\text{Ca}_{\text{act}}$ between the unstimulated basal level and the sustained level obtained 10 min after addition of peptides was taken to compare the calcium response elicited by Pep-13 and structural analogs in aequorin-transformed parsley cells.

$^b$Numbers in parentheses represent the absolute EC$_{50}$ values derived from dose-response curves using aequorin-transformed as well as untransformed parsley cells.

$^c$Bold letters represent alanine substitution sites within Pep-13.
Figure 2. In Vivo Imaging of Pep-13-Induced Changes in [Ca\textsuperscript{2+}]\textsubscript{cyt} in Parsley Cells.

Bioluminescence of reconstituted aequorin in elicitor-treated parsley cells was monitored with a photon-counting video system. Individual images show light emission of parsley cells integrated for the times indicated. Pep-13 (100 nM) was added 30 sec before the start of measurements (i.e., before time zero).

(A) Image series (0 to 160 sec) corresponding to the peak phase observed in luminometric analyses (Figure 1).

(B) Image series (1080 to 1200 sec) corresponding to the plateau phase observed in luminometric analyses (Figure 1).

(C) Integration of light emission over the time course of the experiment (0 to 1200 sec).

(D) Bright-field image of parsley cells used in this experiment. Bar = 1.2 mm.
Elicitor-Induced Increase in Parsley $[\text{Ca}^{2+}]_{\text{cyt}}$

Phase 1 but left phase 2 virtually unaffected (Figure 5A). Importantly, in the presence of RR, elicitor-treated parsley cells maintained the ability to produce phytoalexins, thus providing further evidence that phase 2 but not phase 1 of the $[\text{Ca}^{2+}]_{\text{cyt}}$ response is essential for activating the defense-associated reactions. This also suggests that Pep-13-induced sustained $[\text{Ca}^{2+}]_{\text{cyt}}$ increases may not be due to release from internal RR-sensitive stores.

Like RR, neomycin, an inhibitor of IP$_3$-releasing phospholipase C (Gabev et al., 1989), partially blocked phase 1 but not phase 2 of the Pep-13-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ response (Figure 5B). $\text{Ca}^{2+}$ release through IP$_3$-gated $\text{Ca}^{2+}$ channels is therefore also unlikely to contribute to the elicitor-induced sustained increases of $[\text{Ca}^{2+}]_{\text{cyt}}$ associated with activation of pathogen defense. As shown in Figure 5B, another phospholipase C antagonist, U-73122 (Smallridge et al., 1992), did not inhibit the elicitor-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ response. Both neomycin and RR strongly inhibited a mastoparan-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ response (not shown), which is assumed to reflect release from internal stores. We therefore conclude that this experiment probably probed the actual intracellular pathways for $\text{Ca}^{2+}$ release. Similarly, neomycin blocked a cold shock-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ response, which is believed to result partially from the release of intracellular $\text{Ca}^{2+}$.

In summary, our findings strongly suggest that sustained increases of $[\text{Ca}^{2+}]_{\text{cyt}}$ in elicitor-treated parsley cells are predominantly the result of continuous $\text{Ca}^{2+}$ influx through plasma membrane $\text{Ca}^{2+}$ channels.

$[\text{Ca}^{2+}]_{\text{cyt}}$ Constitutes an Early Element of the Signaling Cascade Triggering Pathogen Defense in Parsley

A variety of pharmacological effectors of signal transduction pathways in eukaryotic cells were used to dissect the Pep-13-activated sequence of cellular responses in parsley. Major emphasis was put on elucidating the role of the elicitor-induced increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ during activation of defense-associated responses, such as production of ROS and phytoalexins.

Heterotrimeric GTP binding proteins (G-proteins) are assumed to link cell surface receptors with intracellular effectors (Bischoff et al., 1999). Administration of the G-protein activator mastoparan to parsley cells yielded concentration-dependent large, rapid, and transient $[\text{Ca}^{2+}]_{\text{cyt}}$ responses (Figure 6). Mas-7, a mastoparan analog with fivefold more biological activity than mastoparan (Higashijima et al., 1990), evoked a substantially larger $[\text{Ca}^{2+}]_{\text{cyt}}$ transient peak than did mastoparan, whereas the inactive mastoparan analog Mas-17 stimulated only a small increase in $[\text{Ca}^{2+}]_{\text{cyt}}$. Our findings suggest that G-protein–activated signaling pathways may function in parsley. However, the $[\text{Ca}^{2+}]_{\text{cyt}}$ signature response stimulated by mastoparan greatly differed from that induced by the elicitors Pep-13, P. sojae glycoprotein, and harpin (Figures 1A and 3A). Consistently, moreover, unlike these elicitors, mastoparan was incapable of activating ROS and phytoalexin production and did not interfere with Pep-13-induced pathogen defense responses.

**Figure 3.** Effect of Abiotic and Biotic Stimuli on $[\text{Ca}^{2+}]_{\text{cyt}}$ Concentrations in Parsley Cells.

Bioluminescence of reconstituted aequorin was monitored in parsley cells treated with various stimuli at the times indicated (arrows). Numbers in parentheses represent amounts of phytoalexins produced by parsley cells treated as indicated relative to those produced by cells treated with 100 nM Pep-13.

- **A** Addition of elicitors stimulating phytoalexin production in parsley cells. The P. sojae-derived 42-kD cell wall glycoprotein, recombinant P. syringae pv phaseolicola–derived harpin, and the polyene antibiotic amphotericin B were added at concentrations sufficient to stimulate elicitor-specific maximum phytoalexin production in parsley cells.
- **B** Addition of elicitors of plant defense-associated responses incapable of stimulating phytoalexin production in parsley cells. Synthetic N-acetylchitoheptaose, a synthetic 15-mer fragment of bacterial flagellin, and purified $\beta$-megaspermin from P. megasperma were added at concentrations at least 10-fold greater than those reported to induce defense-associated responses in various plants.
- **C** Cold shock was applied by adding an equal volume of ice-cold culture medium to parsley cells.
The G-protein activator, cholera toxin, failed to induce any Pep-13–specific response, including the [Ca\textsuperscript{2+}]\textsubscript{cyt} response (not shown). Moreover, cAMP, an element of a G-protein–activated signaling cascade, or cGMP, a product of receptor guanylyl cyclase activity, which is known to activate cyclic nucleotide–gated ion channels (both compounds tested as membrane-permeable dibutyryl derivatives), did not activate a [Ca\textsuperscript{2+}]\textsubscript{cyt} response. In summary, although involvement of G-proteins in pathogen defense signaling has been reported for tomato cells treated with a Cladosporium fulvum–derived elicitor (Blumwald et al., 1998, and references therein), our experiments do not support a role of G-protein–activated signaling pathways in transmitting the elicitor signal in parsley.

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At concentrations previously reported to block chloride efflux, oxidative burst, MAP kinase activation, and phytoalexin production in parsley cells (Jabs et al., 1997; Ligterink

**Figure 4.** Inhibition of Pep-13–Induced Cellular Responses in Parsley Cells by Modulation of Ca\textsuperscript{2+} Influx across the Plasma Membrane.

(A) Parsley cells incubated in culture medium (containing 1 mM CaCl\textsubscript{2}) were challenged with 100 nM Pep-13 at the time point indicated (thick arrow), and changes in [Ca\textsuperscript{2+}]\textsubscript{cyt} were recorded. The heavy line represents recordings obtained from parsley cells treated with Pep-13 only. The thin line represents recordings obtained from parsley cells treated first with the Ca\textsuperscript{2+} chelator BAPTA (4 mM) 5 min before the addition of Pep-13 and then with an equal volume of ice-cold culture medium (cold shock) as indicated (arrowhead). The thin lines descending from the Pep-13 line represent recordings obtained from parsley cells treated with 4 mM BAPTA at the times designated by thin arrows.

(B) Recording of [Ca\textsuperscript{2+}]\textsubscript{cyt} concentrations in parsley cells treated with BAPTA (4 mM) 8 min before the addition of 10 mM CaCl\textsubscript{2} (arrow). The cells were subsequently treated with 100 nM Pep-13 (heavy line) or water (thin line) at the time indicated (arrowhead).

(C) Dependence of the Pep-13–induced increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} (filled squares) and oxidative burst (open squares) on extracellular [Ca\textsuperscript{2+}]. To progressively decrease the amount of extracellular free [Ca\textsuperscript{2+}], parsley cells were incubated with increasing amounts of BAPTA (0 to 4 mM). Extracellular free [Ca\textsuperscript{2+}] was calculated by using MaxChelator software (version 2.5 for Windows; http://www.stanford.edu/~cpatton/) (Bers et al., 1994). Data points represent means of four independent experiments. Error bars indicate SD.

(D) Changes in [Ca\textsuperscript{2+}]\textsubscript{cyt} were recorded from parsley cells treated with the Ca\textsuperscript{2+} channel inhibitor GdCl\textsubscript{3} at the concentrations indicated 5 min before the addition of water (dotted line) or 100 nM Pep-13 (as indicated) at the time noted (arrow). The solid line represents recordings obtained from parsley cells treated with Pep-13 only. Numbers in parentheses indicate the amounts of phytoalexins produced by parsley cells treated as indicated relative to those produced by cells treated with 100 nM Pep-13.
et al., 1997) (Figure 7A), use of the chloride channel inhibitors anthracene-9-carboxylate (A-9-C) and 5-nitro-2-(3-phenylpropylamino)benzoate (NPPB) inhibited phase 1 but not phase 2 of the Pep-13-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) response. The elicitor-induced \(\text{Ca}^{2+}\) influx giving rise to sustained increases in \([\text{Ca}^{2+}]_{\text{cyt}}\) may therefore act upstream of elicitor-induced \(\text{Cl}^-\) channels, which themselves have been shown to be essential for activation of pathogen defense by Pep-13 (Jabs et al., 1997).

The serine/threonine protein kinase inhibitors K-252a (Figure 7B) and staurosporine (not shown) completely blocked both phases of the Pep-13-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) response and ROS production in parsley cells. In contrast, the tyrosine kinase inhibitors genistein and lavendustin did not affect either response (not shown), suggesting that the Pep-13 receptor is unlikely to belong to the tyrosine kinase family of plasma membrane receptors. Inhibitors of protein phosphatase 1 (calyculin A, tautomycin) and 2A (okadaic acid, cantharidin) blocked neither the \([\text{Ca}^{2+}]_{\text{cyt}}\) response nor ROS production in elicited parsley cells (not shown) at concentrations (1 \(\mu\)M) reported to abolish protein phosphatase activity in plant cells (Sopory and Munshi, 1998).

Receptor-Mediated Activation of the \([\text{Ca}^{2+}]_{\text{cyt}}\) Response in Elicitor-Treated Parsley Cells

Activation of elicitor-induced reactions in parsley cells is mediated through binding of Pep-13 to a 100-kD plasma membrane receptor protein (Nürnberger et al., 1994, 1995; Ligtérink et al., 1997; Zimmermann et al. 1997; Nennstiel et al., 1998). To elucidate a possible functional link between Pep-13 perception, activation of \(\text{Ca}^{2+}\) influx, oxidative burst, phytoalexin production, and the Pep-13–induced increase in \([\text{Ca}^{2+}]_{\text{cyt}}\), we investigated a series of structural derivatives of Pep-13. As summarized in Figure 8A and Table 2, the \([\text{Ca}^{2+}]_{\text{cyt}}\) response of elicited parsley cells was activated by those Pep-13 derivatives that efficiently competed for binding of \([^{125}\text{I}]\text{Pep-13}\) to its receptor; they also strongly induced all other responses examined. Replacing Y12 (tyrosine at position 12) with alanine (Pep-13/A12) did not affect the ability of this peptide to bind to the Pep-13 receptor and to stimulate all responses in parsley cells. In contrast, replacing W2 with alanine (Pep-13/A2) rendered this derivative largely inactive with respect to increasing \([\text{Ca}^{2+}]_{\text{cyt}}\), corresponding to observed losses of competitor activity and the inability to trigger \(\text{Ca}^{2+}\) uptake and formation of ROS and phytoalexins. Similarly, deletion of one C-terminal and two N-terminal amino acid residues from Pep-13 (Pep-10) abolished the ability of this derivative to bind to the Pep-13 receptor and to induce increases in \([\text{Ca}^{2+}]_{\text{cyt}}\) and phytoalexin production. Taken together, our data provide evidence that the Pep-13-stimulated increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) is a receptor-mediated process.

Treating apoaequorin-expressing cultured tobacco W38 cells with Pep-13 (0.1 to 1 \(\mu\)M) did not result in increased \([\text{Ca}^{2+}]_{\text{cyt}}\) (not shown). This is in agreement with the absence of a functional Pep-13 binding site in tobacco membranes (not shown) and corroborates previous observations that Pep-13 recognition and subsequent activation of defense responses is specific to parsley (Nürnberger et al., 1995). We further conclude from these experiments that integration of Pep-13 into plant cell membranes in an ionophore-like manner is unlikely.

Desensitization of plant cells to consecutive treatments with the same stimulus is commonly observed (Boller, 1995). To analyze a refractory state in elicitor-treated parsley cells,
Pep-13 was administered at the start of bioluminescence recordings and after the decline of the \([\text{Ca}^{2+}]_{\text{cyt}}\) transient form. As shown in Figure 8B, no additional \([\text{Ca}^{2+}]_{\text{cyt}}\) response was evoked in cells that had been pretreated with Pep-13. Thus, addition of elicitor rendered the system refractory to repeated stimulation by the same signal for at least 40 min. Consistently, addition of the Pep-13–harboring \(P.\) sojae 42-kD glycoprotein elicitor to parsley cells pretreated with Pep-13 did not induce an additional increase in \([\text{Ca}^{2+}]_{\text{cyt}}\). Desensitization was stimulus specific, however, because subsequent addition of mastoparan to Pep-13–treated parsley cells activated a second \([\text{Ca}^{2+}]_{\text{cyt}}\) spike (Figure 8B). Loss of elicitor responsiveness is therefore assumed to indicate desensitization of the Pep-13 perception system.

**DISCUSSION**

Stimulus-dependent changes in \([\text{Ca}^{2+}]_{\text{cyt}}\) control a diverse range of cellular functions, including gene regulation and enzyme activation (Clapham, 1995; Parekh and Penner, 1997). Pulses, repetitive oscillations, and sustained plateaus constitute elements of \([\text{Ca}^{2+}]_{\text{cyt}}\) signatures (Dolmetsch et al., 1997, 1998; Li et al., 1998), all of which have been detected in plant cells (Thuleau et al., 1998; Rudd and Franklin-Tong, 1999; Trewavas, 1999). In parsley cell lines stably expressing the apoaequorin gene, we have monitored a biphasic \([\text{Ca}^{2+}]_{\text{cyt}}\) signature in response to elicitor treatment. We provide evidence that receptor-mediated increases in \([\text{Ca}^{2+}]_{\text{cyt}}\) to sustained higher values are required for activating pathogen defense responses in parsley. Hence, \([\text{Ca}^{2+}]_{\text{cyt}}\) is assumed to act as an early second messenger in transcriptional activation of an array of defense-related genes and subsequent phytoalexin production.

**The Elicitor-Induced Increase in Cytoplasmic Free \([\text{Ca}^{2+}]\) in Parsley Cells Is Receptor Mediated**

Three lines of experimental evidence indicate that elicitor-induced increase of \([\text{Ca}^{2+}]_{\text{cyt}}\) is a receptor-mediated process.
Elicitor-Induced Increase in Parsley \([\text{Ca}^{2+}]_{\text{cyt}}\) (Table 2, and Figures 2 and 8). (1) Replacement of W2 by alanine or deletion of terminal residues markedly reduced the ability of these Pep-13 derivatives to trigger the \([\text{Ca}^{2+}]_{\text{cyt}}\) response. These residues were previously reported to be essential for eliciton as well as competitor activity of Pep-13 (Nümberger et al., 1994, 1995; Zimmermann et al., 1997). Likewise, exchange of alanine for Y12 did not affect the ability of Pep-13 to bind to its receptor and to induce defense responses and left intact the ability of this derivative to trigger the \([\text{Ca}^{2+}]_{\text{cyt}}\) response. (2) Similar elicitor concentrations were sufficient to saturate the elicitor receptor and to trigger increases of \([\text{Ca}^{2+}]_{\text{cyt}}\) as well as other plant responses tested (Table 2). (3) Establishment of a refractory state with respect to repeated stimulation by the same signal was observed in parsley cells treated with Pep-13 (Figure 8B). Such a stimulus-specific desensitization of cellular responses is indicative of receptor-mediated activation and has also been reported for cultured tomato cells treated with fungal sterols or chitooligosaccharides (Boller, 1995).

A biphasic \([\text{Ca}^{2+}]_{\text{cyt}}\) signature of two transient peaks was observed in apoaequorin-transformed soybean cells after treatment with a \(P.\ sojae\)-derived \(\beta\)-glucan fraction or a synthetic hepta-\(\beta\)-glucan (Mithöfer et al., 1999). Although these experiments suggest a functional link between the elicitor-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) response and phytoalexin production, as is reported here, the investigators could not demonstrate a receptor-mediated transient increase of \([\text{Ca}^{2+}]_{\text{cyt}}\) as a requirement for activation of pathogen defense. Moreover, hepta-\(\beta\)-glucan concentrations previously reported to saturate the elicitor receptor (Cosio et al., 1990) were insufficient to trigger the \([\text{Ca}^{2+}]_{\text{cyt}}\) response and phytoalexin production. However, the Mithöfer et al. study does not transform luminescence units into calibrated \([\text{Ca}^{2+}]_{\text{cyt}}\) signature. Thus, evidence for receptor-mediated activation of a defined \([\text{Ca}^{2+}]_{\text{cyt}}\) response signaling activation of pathogen defense was not given.

When treated with \(R.\ meliloti\) nodulation factors, alfalfa root hair cells mount a characteristic \([\text{Ca}^{2+}]_{\text{cyt}}\) signature (Ehrhardt et al., 1996). Image analysis of injected fluorescent \(\text{Ca}^{2+}\) indicators reveals oscillating \([\text{Ca}^{2+}]_{\text{cyt}}\) spikes with a mean oscillation period of 60 sec. Felle et al. (1999) reported a consistent, rapid decrease in extracellular \(\text{Ca}^{2+}\) and concomitant increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) in alfalfa root cells treated with \(R.\ meliloti\)-derived Nod factors. This is remarkably similar to early responses of elicitor-treated parsley cells. Because the structural features of Nod factors required to cause nodulation in alfalfa were also essential for stimulating \([\text{Ca}^{2+}]_{\text{cyt}}\) spiking, Ehrhardt et al. (1996) concluded that there was a functional link between both responses. In addition, these findings also suggest a plasma membrane receptor-mediated activation of either response. However, although high- and low-affinity Nod factor binding sites were shown to reside in Medicago truncatula root cell plasma membranes (Gressent et al., 1999), genuine Nod factor receptors mediating establishment of symbiosis remain to be uncovered.

Elicitor-Induced Sustained Increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) Is Causally Involved in Activation of Pathogen Defense Responses in Parsley

Increases in \([\text{Ca}^{2+}]_{\text{cyt}}\) in parsley cells could be observed as early as 40 sec after addition of Pep-13. A large \([\text{Ca}^{2+}]_{\text{cyt}}\) transient peak of \(\sim 1 \mu\text{M}\) declined rapidly, reaching a plateau phase at \(\sim 250\) to \(350 \text{nM}\) \([\text{Ca}^{2+}]_{\text{cyt}}\) (Figure 1A). In vivo
imaging of aequorin activity revealed steady light emission from small parsley cell clusters during the course of the experiment (Figure 2), suggesting that there is no repeated spiking of [Ca\(^{2+}\)]\(_{cyt}\) in individual cells. In particular, sustained values for [Ca\(^{2+}\)]\(_{cyt}\) seem to be maintained continuously in elicitor-treated parsley cells. However, because the minimum interval for light signal integration was 5 sec, asynchronously responding cells of high-frequency [Ca\(^{2+}\)]\(_{cyt}\) oscillations with mean period times of less than this are possible. On the other hand, studies on stimulus-induced [Ca\(^{2+}\)]\(_{cyt}\) oscillations in plant cells noted mean period times between 15 sec and several minutes (Campbell et al., 1996; Ehhrhardt et al., 1996; Calder et al., 1997; Staxen et al., 1999), which the technology applied would have been sufficient to detect.

Experimental evidence indicates that Pep-13-induced sustained increases in [Ca\(^{2+}\)]\(_{cyt}\) and activation of pathogen defense in parsley are functionally inseparable. (1) Use of elicitors of pathogen defense in parsley (P. sojae-derived glycoprotein elicitor harboring Pep-13, harpin, amphotericin B) revealed that only the second phase of the [Ca\(^{2+}\)]\(_{cyt}\) response, the prolonged increase in [Ca\(^{2+}\)]\(_{cyt}\), was required for phytoalexin production (Figure 3A). Stimuli triggering only the rapid [Ca\(^{2+}\)]\(_{cyt}\) transient peaks—chitosoligosaccharides, a flagellin fragment, mastoparan, nystatin, cold shock—failed to induce sustained increases in [Ca\(^{2+}\)]\(_{cyt}\) and phytoalexin production (Figures 3B and 3C). (2) Pep-13 concentrations that were insufficient to trigger the [Ca\(^{2+}\)]\(_{cyt}\) transient response were sufficient to stimulate both the plateau phase and 80% of the phytoalexins produced at greater Pep-13 concentrations (Figure 1B). (3) The Ca\(^{2+}\) channel inhibitors Gd\(^{3+}\) and RR abolished the Pep-13-induced [Ca\(^{2+}\)]\(_{cyt}\) spike at 100 µM but only slightly affected the [Ca\(^{2+}\)]\(_{cyt}\) plateau value and phytoalexin production (Figures 4D and 5A). (4) Reduction of extracellular Ca\(^{2+}\) by increasing concentrations of BAPTA resulted in gradual inhibition of the ability of Pep-13-treated parsley cells both to mount sustained increases in [Ca\(^{2+}\)]\(_{cyt}\) and to produce ROS and phytoalexins. Similarly, removal of Ca\(^{2+}\) from the culture medium by several washes of cells with Ca\(^{2+}\)-free medium markedly reduced the elicitor-induced [Ca\(^{2+}\)]\(_{cyt}\) response (not shown), the oxidative burst, the transcriptional activation of defense-related genes, and phytoalexin production in parsley cells (Nürnberger et al., 1994; Jabs et al., 1997). (5) Elicitor treatment of parsley cells in the presence of the Ca\(^{2+}\) channel blockers Gd\(^{3+}\) (Figure 4D) and La\(^{3+}\) or the protein kinase inhibitors (Figure 7B) inhibited the increase in [Ca\(^{2+}\)]\(_{cyt}\) and production of ROS and phytoalexins. (6) Complementary gain-of-function experiments using amphotericin B revealed that artificially increasing [Ca\(^{2+}\)]\(_{cyt}\) to sustained values triggered both the [Ca\(^{2+}\)]\(_{cyt}\) response and the production of ROS and phytoalexins in the absence of elicitor (Figure 3A; Jabs et al., 1997). (7) Identical structural properties of Pep-13 were found to be required for efficient stimulation of the [Ca\(^{2+}\)]\(_{cyt}\) plateau, of the oxidative burst, and of phytoalexin production (Table 2). (8) Initial changes in [Ca\(^{2+}\)]\(_{cyt}\) clearly preceded ROS production and phytoalexin formation in elicitor-treated parsley cells (Figure 1A; Nürnberger et al., 1994).

Taken together, these findings indicate that the sustained increase of [Ca\(^{2+}\)]\(_{cyt}\) rather than a single transient increase in [Ca\(^{2+}\)]\(_{cyt}\) may encode signal specificity toward activation of pathogen defense. The physiological implication of the large [Ca\(^{2+}\)]\(_{cyt}\) transient triggered by Pep-13, however, remains elusive.

A similar [Ca\(^{2+}\)]\(_{cyt}\) response has been implicated in plant adaptation to abiotic stress. Ozone fumigation of Arabidopsis plants elicited a biphasic [Ca\(^{2+}\)]\(_{cyt}\) response with a prolonged second phase required for transcriptional activation of a glutathione S-transferase gene (Clayton et al., 1999). Similarly, microinjection of cADP ribose into Commelina communis guard cells induced a sustained increase in [Ca\(^{2+}\)]\(_{cyt}\) and resulted in stomatal closure (Leckie et al., 1998). A biphasic [Ca\(^{2+}\)]\(_{cyt}\) response involving an initial transient peak followed by a second, prolonged, slow increase was also detected in Arabidopsis roots during cold acclimation (Plieth et al., 1999).

The importance of defined [Ca\(^{2+}\)]\(_{cyt}\) signals for initiating different response pathways is intriguingly exemplified by activation of the inflammatory response in human B lymphocytes. The proinflammatory transcriptional regulators NF-κB and c-Jun N-terminal kinase (JNK) are activated by a [Ca\(^{2+}\)]\(_{cyt}\) spike, whereas another essential transcriptional regulator, nuclear factor-activated T cell (NFAT), is activated by a [Ca\(^{2+}\)]\(_{cyt}\) plateau (Dolmetsch et al., 1997). Moreover, the [Ca\(^{2+}\)]\(_{cyt}\) oscillation frequency was shown to modulate gene expression in these cell lines (Dolmetsch et al., 1998; Li et al., 1998). Apparently, downstream effectors decode complex [Ca\(^{2+}\)]\(_{cyt}\) signatures, which probably constitute a mechanism by which the multifunctional second messenger [Ca\(^{2+}\)]\(_{cyt}\) maintains specificity of signaling cascades. This view is supported by De Koninck and Schuman (1998), who reported that Ca\(^{2+}\)/calmodulin-dependent protein kinase II can decode the frequency of Ca\(^{2+}\) spikes into distinct amounts of its activity in vitro.

**Elicitor-Induced Increase in [Ca\(^{2+}\)]\(_{cyt}\) and Activation of Pathogen Defense Responses Requires Extracellular Ca\(^{2+}\)**

Use of pharmacological effectors provided evidence that elicitor-induced sustained concentrations of [Ca\(^{2+}\)]\(_{cyt}\) may predominantly reflect the influx of extracellular Ca\(^{2+}\). Chelation of extracellular Ca\(^{2+}\) by membrane-impermeable BAPTA (Figures 4A and 4C) or EGTA completely abolished the increase in [Ca\(^{2+}\)]\(_{cyt}\) and the activation of downstream responses in parsley cells, such as the oxidative burst (Figure 4C). In addition, only under experimental conditions in which an inwardly directed [Ca\(^{2+}\)] gradient was maintained were parsley cells sensitive to elicitor treatment. Because BAPTA abrogated the [Ca\(^{2+}\)]\(_{cyt}\) response also when added after the elicitor, a continuous Ca\(^{2+}\) influx appears to be required for
maintaining increased \([Ca^{2+}]_{cyt}\). To ensure a plateau value for \([Ca^{2+}]_{cyt}\) extrusion or sequestration of \(Ca^{2+}\) from the cytoplasm may therefore accompany elicitor-induced \(Ca^{2+}\) entry into the cytoplasm. Lanthanide inhibitors of plasma membrane \(Ca^{2+}\) channels blocked the elicitor-induced increase in \([Ca^{2+}]_{cyt}\) and the plant defense responses as well (Figure 4). The same inhibitors had previously also been found to block activation by Pep-13 of a parsley plasma membrane \(Ca^{2+}\)-permeable ion channel (Zimmermann et al., 1997). Likewise, treatment of parsley cells with the ionophore amphotericin B resulted in a \(Ca^{2+}\) influx, an increase in \([Ca^{2+}]_{cyt}\), the transcriptional activation of defense-related genes, and production of ROS and phytoalexins in the absence of elicitor (Jabs et al., 1997; Figure 3A). Furthermore, the attempted modulation of phospholipase C activity by neomycin, with the subsequent \(Ca^{2+}\) release from internal stores, and the inhibition by RR of RR-sensitive intracellular \(Ca^{2+}\) release channels failed to inhibit elicitor-induced sustained increases in \([Ca^{2+}]_{cyt}\) or activation of plant defense responses (Figure 5).

Increases in \([Ca^{2+}]_{cyt}\) in Nod factor-treated alfalfa root hair cells or in elicitor-treated soybean cells were also found to result mainly from a stimulus-induced \(Ca^{2+}\) influx through the plasma membrane (Felle et al., 1999; Mithöfer et al., 1999). In contrast, the increases of \([Ca^{2+}]_{cyt}\) monitored in plant cells in response to anoxia, cold shock, drought, or salinity have been ascribed to both a \(Ca^{2+}\) release from internal stores and an influx of external \(Ca^{2+}\) (Subbaiah et al., 1994; Haley et al., 1995; McAinsh et al., 1995; Knight et al., 1996, 1997). Thus, just as in animal cells, two major pathways for \(Ca^{2+}\) release into the cytoplasm may be operative in plant cells (Clapham, 1995; Parekh and Penner, 1997; Thuleau et al., 1998; Rudd and Franklin-Tong, 1999; Ca\(^{2+}\)-influx directly through plasma membrane \(Ca^{2+}\) channels, and \(Ca^{2+}\)-entry by second messenger-induced depletion of intracellular stores followed by store replenishment by way of \(Ca^{2+}\)-release-activated \(Ca^{2+}\) currents across the plasma membrane.

Numerous \(Ca^{2+}\) channels have been identified in plant cells, but their physiological roles and probable cooperation in response to various stimuli are little understood. Challenging future tasks will be to understand how different \(Ca^{2+}\) channels contribute to signal-specific \([Ca^{2+}]_{cyt}\) signatures and how \(spatio\)temporal alterations in \([Ca^{2+}]_{cyt}\) can activate downstream effectors, such as \(Ca^{2+}\)- or \(Ca^{2+}\)/calmodulin-dependent protein kinases (Blumwald et al., 1998; Thuleau et al., 1998; Rudd and Franklin-Tong, 1999; Trewavas, 1999). chemistry as described by Nennstiel et al. (1998). Pep-13/A12 and Pep-10 were purchased from Kem-En-Tec A/S (Copenhagen, Denmark), and Mas-7 was from Calbiochem (Bad Soden, Germany). The Phytophthora sojae 42-kD cell wall glycoprotein elicitor was purified according to Nünberger et al. (1994). Purified recombinant Pseudomonas syringae pv phaseolicola harpin was from Justin Lee (IPB, Halle, Germany). The Phytophthora megasperma elicin \(\beta\)-megasperm was provided by Serge Kaufmann (INRA, Strasbourg, France). N-acetylcysteine was from Naoto Shibuya (University of Tsukuba, Tsukuba, Japan), and N-acetylcysteine was from Jürg Felix (FM, Basel, Switzerland). Inhibitors were purchased from Alexis (Grünberg, Germany), anthracene-9-carboxylate (A-9-C) and N-acetylcysteine from Sigma (Deisenhofen, Germany), amphotericin B from Calbiochem, and EGTA, CaCl\(_2\), and GdCl\(_3\) as well as high-purity chemicals for in vitro \([Ca^{2+}]_{cyt}\) calibration from Fluka (Deisenhofen, Germany). 1,2-Bis(aminophenoxy)ethane-\(N,N,N'\)-tetra-acetic acid (BAPTA), coelenterazine, and calcium calibration buffers were supplied by Molecular Probes (Leiden, The Netherlands).

### Plant Cell Culture and Elicitor Treatment

Dark-grown cell suspension cultures of Petroselinum crispum were used for elicitor treatment and reconstitution of aequorin 5 days after inoculation. Culture maintenance and quantification of reactive oxygen species (ROS) and furanocoumarin phytoalexin production were performed according to Nünberger et al. (1994). Cell viability was determined by double-staining with fluorescein diacetate and propidium iodide (Jabs et al., 1997) 30 min or 24 hr after treatment, respectively. Tobacco W38 cell suspensions stably transformed with aapoaequorin were obtained from Phil Low (Purdue University, West Lafayette, IN) and maintained as described (Chandra et al., 1997).

### Aequorin Constructs and Parsley Cell Transformation

Three-day-old parsley cells were transformed as described (Frohnmeyer et al., 1999) by particle bombardment with a plasmid carrying aequorin cDNA inserted between a double cauliflower mosaic virus 35S promoter, a tobacco mosaic virus \(\Gamma\)-element, and a 35S terminator (pAEQ/HYG). This plasmid was derived from pGNAEQ/NEO2 (Mithöfer et al., 1999) by replacing the cassette for the selectable marker NPTII with a hygromycin-resistance gene (HP7) excised from plasmid pGL2 (Mithöfer et al., 1999). Plasmids pGNAEQ/NEO2 and pGL2 were kindly provided by Gunther Neuhaus (University of Freiburg, Germany).

### Aequorin Reconstitution and Bioluminescence Measurement

In vivo reconstitution of aequorin was initiated by adding 3 \(\mu\)L of coelenterazine (5 mM stock solution in methanol) to 3 mL of 5-day-old aequorin-transgenic parsley cells. Cells were adjusted to 60 mg (fresh weight) mL\(^{-1}\) culture medium supplemented with 1 mM CaCl\(_2\). Subsequently, cells were incubated in the dark under continuous shaking (for 6 hr or overnight, at 26°C and 120 rpm).

Bioluminescence counts from 100-\(\mu\)L cell culture aliquots were recorded at 10-sec intervals (recorded as average relative light units (RLUs)/sec) with a digital luminometer (Lumat LB9501; Berthold, Bad Wildbad, Germany). At the end of each experiment, the remaining aequorin was discharged by adding two volumes of 37.5 mM CaCl\(_2\)/15% ethanol (v/v). The amount of aequorin consumed at each time

### METHODS

#### Materials

The peptides Pep-13, Pep-13/A2, mastoparan, mastoparan-17, and flagellin-15 were synthesized by use of fluorenly methoxycarbonyl
point of the experiment never exceeded 5% of the total remaining aequorin. Alternatively, bioluminescence assays were performed with a microplate luminometer (Luminoskan Ascent, version 2.4; Labsystems, Frankfurt/Main, Germany) under continuous shaking of cell suspensions (300-μL aliquots in 48-well plates, measured every 13 sec). No differences in reproducibility were observed between the two bioluminescence monitoring devices used.

In vivo calcium imaging of aequorin activity in parsley cells was performed with a C2400-40H intensified (CCD) Charge-Coupled Device Camera device (Hamamatsu Photonics GmbH Deutschland, Herrsching, Germany) equipped with a Nikon lens system (f = 35 mm, 40-mm-long extension tube; Nikon Deutschland, Düsseldorf, Germany) and the operating software HiPic 5.1. Parsley cells (5 × 10^6 in 100 μL) were placed on a microscope slide, treated with elicitor, and subjected to photon counting in the dark. Bioluminescence was recorded in slice mode in dynamic photon-counting format.

Calibration of Calcium Measurements

To transform bioluminescence counts into absolute [Ca^{2+}] values, we established a calibration curve from in vitro measurements of protein extracts made from pAEQ/HYG-expressing parsley cells and a series of buffers with various [Ca^{2+}]. Transgenic parsley cells were washed with calcium-free buffer, frozen in liquid nitrogen, and homogenized in reconstitution buffer: 20 mM Tris/HCl, pH 7.4, 150 mM KCl, 5 mM EDTA, 0.1 mM EGTA, 50 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation (5 min, 4°C, 12,100g) the supernatant was utilized for in vitro reconstitution of aequorin (3 hr, 4°C) in the presence of 0.1% gelatin and 2.5 μM coelenterazine.

Light production was measured in buffers (10 mM Heps, pH 7.2, 100 mM KCl, 1 mM MgSO_4 with known [Ca^{2+}] (Molecular Probes calcium calibration buffer kit). [Ca^{2+}] values were subsequently calculated as rate constants from the total aequorin activity assessed at saturating concentrations of Ca^{2+} according to Cobbold and Lee (1991). Rate constants represent bioluminescence counts monitored per second divided by the total remaining bioluminescence counts.

All reagents were applied to lysates containing recombinant aequorin to examine the direct effects of the reagents on aequorin luminescence. None of these substances interfered with aequorin luminescence.

Extracellular free [Ca^{2+}] in the presence of the chelator BAPTA was calculated using MaxChelator software (version 2.5 for Windows; http://www.stanford.edu/~cpatton/) (Bers et al., 1994).

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REFERENCES


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