Effects of Feeding Spodoptera littoralis on Lima Bean Leaves. I. Membrane Potentials, Intracellular Calcium Variations, Oral Secretions, and Regurgitate Components

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Membrane potentials ($V_m$) and intracellular calcium variations were studied in Lima bean (Phaseolus lunatus) leaves when the Mediterranean climbing cutworm (Spodoptera littoralis) was attacking the plants. In addition to the effect of the feeding insect the results showed that the early events upon herbivore attack were: a) a strong $V_m$ depolarisation at the bite zone and an isotropic wave of $V_m$ depolarisation spreading throughout the entire attacked leaf; b) a $V_m$ depolarisation observed for the regurgitant but not with volicitin [$N$-(17-hydroxy-linolenoyl)-Gln] alone; c) an enhanced influx of Ca$^{2+}$ at the very edge of the bite, which is halved, if the Ca$^{2+}$ channel blocker Verapamil is used. Furthermore, the dose-dependence effects of $N$-acyl Gln conjugates-triggered influx of Ca$^{2+}$ studied in transgenic aequorin-expressing soybean (Glycine max) cells, showed: a) a concentration-dependent influx of Ca$^{2+}$; b) a configuration-independent effect concerning the stereochemistry of the amino acid moiety; c) a slightly reduced influx of Ca$^{2+}$ after modification of the fatty acid backbone by functionalization with oxygen and; d) a comparable effect with the detergent SDS. Finally, the herbivore wounding causes a response in the plant cells that cannot be mimicked by mechanical wounding. The involvement of Ca$^{2+}$ in signaling after herbivore wounding is discussed.

Several plant species, including Lima bean (Phaseolus lunatus), when attacked by herbivores emit volatiles that attract natural predators of the damming insects. This signaling by the plant to higher trophic levels has been interpreted as the plant’s cry for help (Dicke and Sabelis, 1992; Turlings et al., 1995; DeMoraes et al., 1998) and involves at least three different levels of trophic interaction (Agrawal, 2000). Thus, volatile plant compounds released in response to insect feeding are directly associated with the feeding herbivore, which allows the plant to differentiate between mechanical wounding and wounding caused by the chewing insect (Paré et al., 1998). In fact, upon herbivore attack, plants activate a series of genes indicating that feeding insects are able to elicit and up-regulate defense in plants (Thaler, 1999; Baldwin et al., 2001; Schittko et al., 2001; Hui et al., 2003). Furthermore, uninfested Lima bean leaves activate defense genes when exposed to volatiles from conspecific leaves infested with herbivores, e.g. spider mites (Tetranychus urticae), but not when exposed to volatiles from artificially wounded leaves (Arimura et al., 2000a). Expression of these genes very often requires some of the early events in the signal transduction cascade such as calcium influx and protein phosphorylation/dephosphorylation, jasmonate, ethylene, and salicylates (Blumwald et al., 1998; Poppy, 1999; Arimura et al., 2000a, 2002; Winz and Baldwin, 2001). The first events following leaf wounding and introduction of the herbivore elicitors are not well understood, but in several cases the activation of the octadecanoid-signaling cascade has been demonstrated (Koch et al., 1999, and references cited therein). However, damaging leaves also generates a first-line of cell reactions involving both electrical signals and production of reactive oxygen species.

Plants are continuously interacting with the external world. The coordination of internal processes and their balance with the environment are connected with the excitability of plant cells. The primary candidate for intercellular signaling in higher plants is the stimulus-induced change in plasma membrane potential ($V_m$; Labady et al., 2002). $V_m$ are the result of an imbalance in the quantity of cations and anions across biological membranes. However, the binding of many plant natural products to membranes causes conformational changes in the ion channels and membrane-bound proteins as well as formation of new ion channels or pores, increasing or decreasing ion flow (Warber, 1998; Engelberth et al., 2001; Maffei et al., 2001). Recent electrophysiological studies allowed to identify the involvement of a rapid electrical signal in root to shoot communications in Sorghum bicolor (Mishra et al., 2001), whereas in soybean (Glycine

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max) phloem appears to participate in the transmission of fast root-to-shoot action potentials upon stress (Shvetsova et al., 2001).

The \( V_m \) of the plasma membrane, which lies in the range of \(-120\) to \(-200\) mV in plant cells, may be shifted either to more negative (hyperpolarization) or to more positive values (depolarization) in response to various biotic or abiotic stresses. In plant cells \( \text{Ca}^{2+} \) plays a key physiological role as intracellular second messenger. It is especially important for the maintenance of cellular homeostasis and signal transduction pathways (Evans et al., 1991; Piñeros and Tester, 1997; Roh et al., 1998).

Among the \( \text{Ca}^{2+} \)-permeable channels characterized in the plasma membrane of plant cells, voltage-activated \( \text{Ca}^{2+} \) channels may contribute to calcium signaling (Thulieau et al., 1998). Interestingly, the plant plasma membrane contains at least three distinct classes of voltage-activated \( \text{Ca}^{2+} \) channels stimulated by hyperpolarization (Gelli and Blumwald, 1997), depolarization (Piñeros and Tester, 1995) and voltage insensitive channels (White, 2000), respectively.

Recently, it has been demonstrated that when plants are wounded, jasmonate is synthesized and employed as a long-distance signal that activates the wound response program in unwounded leaves (Stratmann, 2003). Also the floral scent methyl jasmonate has been demonstrated to be involved in gene-activation control and systemic long-distance signaling (Cheong and Choi, 2003). Much less is known about the individual components from the salivary secretions of the feeding insects that lead to the up-regulation of the jasmonate signaling. Volicitin, \( N-(17\text{-hydroxylinolenoyl})-\text{Gln} \), a major component from the regurgitant of \textit{Spodoptera littoralis} Boisd. larvae, has been shown to induce a systemic release of volatiles from maize (\textit{Zea mays}) plants (Alborn et al., 1997) and \( N\)-acyl Glu, for example \( N\)-linolenoyl-Glu, from the regurgitant of the tobacco hornworm (\textit{Manduca sexta}) elicit nicotine biosynthesis in tobacco (\textit{Nicotiana tabacum}) leaves. Both compound types are highly surface active amphiphiles and were shown to act via the jasmonate pathway (Halitschke et al., 2001; Schmelz et al., 2003).

Figure 1. Lima bean leaf \( V_m \) values as a function of distance from the bite zone 15 min after herbivore damage. The histogram superimposed on Lima bean leaf wounded by a larva of \textit{S. littoralis} represents \( V_m \) values (and sd) measured at increasing distances from the bite zone. The dotted line (and its sd) represents the average \( V_m \) value from a mechanically wounded Lima bean leaf. In the close vicinity of the bite zone (up to 1.5 mm) there is a strong drop in the \( V_m \) (depolarization), whereas at about 2.5 to 3 mm from the bite zone an increase of \( V_m \) is observed (hyperpolarization). About 6 mm from the bite zone throughout all leaf there is a constant \( V_m \) depolarization.

Effects of Feeding \textit{Spodoptera littoralis} on Lima Bean Leaves

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Other molecules are also involved in wound responses and the most studied are \( \text{H}_2\text{O}_2 \) (Orozco-Cárdenas et al., 2001; Pellinen et al., 2002), salicylic acid (Shah, 2003), and ethylene (Winz and Baldwin, 2001).

So far, most of the work on plant-insect interaction has been done on gene activation and evaluation of the various elements of the signaling pathway in plant cells. To our knowledge almost nothing is known about the early signals upon herbivore attack at the membrane level and the connections between individual components of the salivary secretions of the insects and the subsequent up-regulation of the octadecanoid biosynthesis. In this work we present data on the early events during feeding of *S. littoralis* on Lima bean leaves and on the effect of individual, especially the surface active spit components, on changes in the \( V_m \) and their correlation with intracellular calcium variations.

**RESULTS**

**Effect of Feeding *S. littoralis* on Lima Bean Leaf \( V_m \)**

In plants unable to produce constitutive defenses such as glandular trichomes filled with irritating essential oils or morphological structures such as thorns and stinging hairs, the attack of herbivores is a devastating experience, leading to the destruction of fed tissues. However, some plants react to this event by producing volatile organic compounds able to reduce wounding by attracting predators of the attacking herbivore. This type of induced defense requires recognition of the attacking herbivore and the specific interactions of the plant physiology with the oral secretions. The primary candidate for intercellular signaling in higher plants is the stimulus-induced (herbivore wounding) change in \( V_m \) (Shvetsova et al., 2001).

The results of the measurement of \( V_m \) after mechanical wounding and herbivore attack indicate a specific response of the leaf tissue. Lima bean leaf \( V_m \) varies according to the cell type. Preliminary tests on intact leaves allowed evaluating the average \( V_m \) of epidermal, guard cell, palisade, and spongy parenchyma cells. Epidermal cells have an average \( V_m \) of \(-50\) mV (±5.7 mV), guard cells have an average \( V_m \) of \(-200\) mV (±12.2 mV), palisade cells have an average \( V_m \) of \(-140\) mV (±9.8 mV), and spongy parenchyma cells have an average \( V_m \) of \(-100\) mV (±10.5 mV). Different trials demonstrated that Lima bean palisade cells are the most responsive cells when leaf tissues are attacked by larvae of *S. littoralis*.

To study the early effects at the bite zone and subsequent signal spreading, \( V_m \) was evaluated at increasing distances from the site of damage. The response was a strong \( V_m \) depolarization in the bite zone, followed by a transient \( V_m \) hyperpolarization and, finally, a constant \( V_m \) depolarization throughout the rest of the attacked leaf. Figure 1 shows the \( V_m \) variations superimposed on the wounded Lima bean leaf tissue. The ordinates represent \( V_m \) expressed in mV, while in the abscissa the bands (and the corresponding histogram bars) represent different distances (and the corresponding \( V_m \) values) from the bite zone. The \( V_m \) of the mechanically wounded leaf (control) is represented by the dashed line. Exponential interpolation shows the trend of \( V_m \) variation. A strong \( V_m \) depolarization was found up to about 1.5 mm from the bite zone, whereas a \( V_m \) hyperpolarization was found at about 2.5 to 3 mm from the bite zone, immediately followed by a second strong \( V_m \) depolarization. \( V_m \) differences from control in the zone from 3.5 to about 6 mm from the bite zone were not significant, but \( V_m \) displayed depolarized values from 6 mm throughout all the attacked leaf (Fig. 1).

The trend of the \( V_m \) variation prompted a series of experiments aimed to better understand the nature and the reasons for this effect. The first attempt was to probe whether the feeding activity of the herbivore was perceived as a \( V_m \) variation even at considerable distances from the bite zone in the same leaf. An intact leaf from a potted plant was fixed to the \( V_m \) apparatus and the \( V_m \) determined. When \( V_m \) reached a constant value *S. littoralis* was allowed to start its feeding activity. Figure 2 depicts \( V_m \) variations as a function of time and distance from mechanically wounded (MW) Lima bean leaf tissue, starting with a potential of about \(-137\) mV, and \( V_m \) from a leaf under attack by *S. littoralis*. It is
evident that feeding activity starts a series of $V_m$ variations eventually leading to $V_m$ depolarization within the first 15 min after the onset of the feeding activity. In particular, when $V_m$ was taken from palisade cells at an average distance of 5 mm a strong and transient hyperpolarization occurred within 5 min after the herbivore bite, followed by a constant depolarization. The same pattern was observed when $V_m$ palisade cell was measured at a distance of 30 mm from the bite zone, but depolarization was higher than in cells at 5 mm distance. Finally, in palisade cells that were 60 mm distant from the bite zone $V_m$ depolarization occurred within 2 to 3 min from the bite event and no hyperpolarization was observed (Fig. 2).

From Figure 2 it is evident that the recognition of the bite activity of $S.\ litoralis$ is quickly perceived in the same leaf at increasing distances from the bite area. However, the attempt to find variations in neighboring leaves (OL) resulted in no obvious variations as did mechanical wounding (MW) on the same leaf (Fig. 2).

**Effect of $S.\ litoralis$ Regurgitate and Regurgitate Components on Lima Bean Leaf $V_m$**

In order to evaluate which molecule may be responsible of $V_m$ variations a series of experiments was carried out using regurgitate (R) collected from larvae previously feeding on Lima bean leaves for 24 h. Perfusion with R caused a $V_m$ depolarization; however, the effect was found not to be linearly linked to concentration. In fact, perfusion with 100 $\mu$g mL$^{-1}$ R depolarized $V_m$ more than perfusion with 250 $\mu$g mL$^{-1}$, but less than perfusion with 500 $\mu$g mL$^{-1}$. Interestingly, when R was washed out with fresh buffer, palisade $V_m$ experienced a hyperpolarization for all concentrations, with an opposite trend as observed during depolarization (Fig. 3).

Since previous studies have demonstrated that R of $S.\ litoralis$ contains several surface active, amphiphilic compounds, especially N-acyl Gln conjugates (Alborn et al., 1997; Pohnert et al., 1999a; Spiteller and Boland,

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**Figure 3.** Effect of $S.\ litoralis$ oral secretions and regurgitants (R) on the $V_m$ of Lima bean palisade cells. At the lowest concentration (100 $\mu$g mL$^{-1}$) R caused an intermediate $V_m$ depolarization when compared to concentrations of 250 and 300 $\mu$g mL$^{-1}$. After washing the tissues with fresh buffer (double arrow) $V_m$ was hyperpolarized at all concentrations, and once again R at 100 $\mu$g mL$^{-1}$ had an intermediate value. The single arrow indicates start of perfusion after $V_m$ stabilization. Metric bars = SD.

**Figure 4.** A, $V_m$ changes after perfusion of Lima bean leaves with 17-R,S-volicitin ($= N$-[17R,S-hydroxylinoleoyl]-L-Gln) and the naturally occurring 17S-volicitin ($= N$-[17S-hydroxylinoleoyl]-L-Gln). At all concentrations, both the racemic mixture and the natural volicitin caused no variation of $V_m$. B, Effect on the $V_m$ of different N-palmitoleoyl-L-Gln concentrations. The highest concentration (300 $\mu$g mL$^{-1}$) had lower $V_m$ hyperpolarization effects than 100 $\mu$g mL$^{-1}$, whereas at 25 $\mu$g mL$^{-1}$ N-palmitoleoyl-L-Gln caused a constant $V_m$ depolarization. C, Effect of different N-linolenoyl-L-Gln concentrations on $V_m$. Clear effects were only caused by concentrations at 100 $\mu$g mL$^{-1}$. A single arrow indicates start of perfusion after $V_m$ stabilization. The double arrow indicates washing with fresh buffer. Metric bars = SD.
Whereas at higher concentration (500 mM) a clear depolarization was observed, even after washing with fresh buffer (double arrow). The single arrow indicates start of perfusion after V_m stabilization. Metric bars = 50.

Figure 5. Effect of increasing concentrations of the detergent SDS on V_m. Lower concentrations (from 50–100 μM mL⁻¹) had no effect, whereas at higher concentration (500 μM mL⁻¹) a considerable V_m depolarization was observed, even after washing with fresh buffer (double arrow). The single arrow indicates start of perfusion after V_m stabilization. Metric bars = 50.

Intracellular calcium variations may depend on both the entry of Ca²⁺ in the cytoplasm upon release from cell organelles and the entry from the apoplasm. Since plant cells respond to extracellular stimuli with changes in cytosolic calcium concentration that ultimately controls many integrated physiological processes (Bush, 1995), the impact of feeding on cytosolic calcium concentration, [Ca²⁺]_c, was investigated. Using the membrane-permeant Ca²⁺-selective fluorescent dye, Fluo-3 AM, the [Ca²⁺]_c were determined by confocal laser scanning microscopy in mechanically- and herbivore-wounded leaf tissue, as well as in tissues treated with linolenoyl-L-Gln. Verapamil, a calcium channel blocker, was additionally used in all experiments. [Ca²⁺]_c, was expressed as the percentage of calcium variation in image analysis where the lowest value (0%) refers to zero fluorescence (value 0 on the 0–256 gray level scale) and the highest values (100%) refer to the maximum fluorescence (value 256 on the 0–256 gray level scale).

Figures 6 and 7 show the results of the experiments performed incubating Lima bean leaves with 5 mM...
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Fluo-3 AM in 50 mM MES buffer both in the presence of 0.5 mM calcium and without calcium in the incubation medium and in the presence or absence of 100 μM of Verapamil. As a general consideration, in all experiments performed in the presence of Ca$^{2+}$ a clear peak of Fluo-3 AM fluorescence was observed between 30 and 200 μm from the bite zone, with a sharp decrease at distances greater than 230 μm (Figs. 6 and 7).

Mechanical wounding caused an influx of Ca$^{2+}$ (Figs. 6D and 7, Al). When Ca$^{2+}$ was absent from the incubation medium (Fig. 7, All) or when Verapamil was present (Fig. 7, All and AIV) a reduced Ca$^{2+}$ influx was observed. The average percentage of fluorescence in mechanically wounded leaves was 25% near the damage in the presence of Ca$^{2+}$ and 5% to 10% without Ca$^{2+}$. An average of 10% to 15% was recorded in the presence of Ca$^{2+}$ at increasing distances from the bite zone, whereas only 5% to 10% was found without Ca$^{2+}$. When mechanically wounded leaves were treated with the larval R, an increase in Fluo-3 AM fluorescence was found in the absence of Ca$^{2+}$ (Fig. 7, BII) whereas in the presence of Verapamil the occurrence of Ca$^{2+}$ increased Fluo-3 AM fluorescence (Fig. 7, BIII). When N-linolenoyl-Gln (100 μg mL$^{-1}$) was given as a 0.5 mM Ca$^{2+}$-containing solution to mechanically wounded leaves, no differences were observed between tissues treated with (Fig. 7, CI) or without (Fig. 7, CII) Ca$^{2+}$ in the medium, and the same was observed for Verapamil (Fig. 7, CIII and CIV). Almost the same results were found when N-linolenoyl-Gln was applied as a Ca$^{2+}$ free solution (Fig. 7, DIII). On the other hand, when larvae were allowed to feed on tissues incubated with Fluo-3 AM, a strong Fluo-3 AM fluorescence was observed. In the presence of Ca$^{2+}$, in both larvae reared on artificial diet (Fig. 7, EI) and larvae reared on Lima bean leaves (Figs. 6A and 7, FI), a sharp and consistent peak of fluorescence was observed up to 100 μm from the bite zone. The absence of Ca$^{2+}$ dramatically decreased Fluo-3 AM fluorescence in both feeding experiments (Figs. 6B, 7EII, and 7FI). When Verapamil was added in the presence of Ca$^{2+}$ (Figs. 6C, 7EIII, and 7FI) it almost halved Fluo-3 AM fluorescence, whereas the absence of Ca$^{2+}$ in Verapamil treated tissues (Fig. 7, EIV and FIV) showed a decreased Fluo-3 AM fluorescence.

Quantification of Ca$^{2+}$-Release by N-acyl Glns in Suspension Cultures of Soybean

Loading of Ca$^{2+}$-sensitive fluorescent probes into plant cells is an essential step to measuring activities of cytoplasmic free Ca$^{2+}$ ions with a fluorescent imaging technique. However, barriers to the loading of the test compounds or the Ca$^{2+}$-sensitive fluorescent dyes could be represented by a low permeability of the cell wall as well as by a massive cuticle. This would allow the penetration of only a limited number of cell layers probably near the infection zone. Thus, in order to study dose dependent effects of N-acyl-Gln-triggered Ca$^{2+}$ concentration changes in the plant cytosol, transgenic soybean suspension cells expressing the Ca$^{2+}$ sensitive aequorin system were used for further experiments (Mithöfer et al., 1999; Mithöfer and Mazars, 2002). Although among independent experiments the maximum values of the [Ca$^{2+}$]c varied to some extent, in a single set of measurements using the same population of suspension cells the same day, the relative activities of the effectors analyzed have always been comparable. The Ca$^{2+}$ response was determined in a concentration-dependent fashion for the most common N-acyl conjugate N-linolenoyl-Gln. For this compound the transiently accumulating [Ca$^{2+}$], appeared to be linearly correlated with the amount of effector applied (Fig. 8A). Moreover, it is interesting to note that the configuration of the amino acid moiety had no effect on the Ca$^{2+}$-response. Both conjugates, containing either L-Gln or D-Gln joined to linolenic acid were able to trigger the Ca$^{2+}$ response (Fig. 8B).

Similar results were observed with the corresponding N-acyl Glus, which are typical constituents of the regurgitant of the tobacco horn worm Manduca sexta (Halitschke et al., 2001). Again, both conjugates of linolenic acid with either D- or L-Glu proved to be active (Fig. 8B). A direct comparison among volicitin, N-linolenoyl Gln, and the recently identified 15,16-epoxyoctadeca-9,12-dienyl-Gln (Spiteller and Boland, 2003a, 2003b) at the same concentration, showed that the nonfunctionalized N-linolenoyl-Gln is the most effective compound (Fig. 8C); however, differences were small. For comparison, we also tested the detergent SDS (25 μg mL$^{-1}$), which showed a highly similar pattern of Ca$^{2+}$-influx (Fig. 8D) demonstrating that the influx of Ca$^{2+}$ is a rather unfocused effect linked to the intrinsic molecular properties of amphiphilic compounds.

DISCUSSION

Plant responses to herbivore attack are complex and involve an array of signals, leading to activation of multiple defenses. Feeding herbivores cause extensive and irreversible wounding along with an introduction of salivary secretions. Both wounding and components from the insects’ secretions have an obvious, but clearly different impact on the plants’ response (Schiffko et al., 2001, and references cited therein). In the model system Nicotiana attenuata and its specialist
Figure 8. Monitoring of cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) changes in soybean cell suspension cultures expressing the Ca\(^{2+}\) sensing aequorin system. A, Enhancement of [Ca\(^{2+}\)]\(_c\) determined upon treatment with increasing concentrations (2, 10, 50, and 200 \(\mu\)g mL\(^{-1}\)) of N-linolenoyl-Gln. B, Comparison of the amino acid configurations (l- or d-Gln and l- or d-Glu, respectively) of the linolenic acid conjugates (50 \(\mu\)g mL\(^{-1}\)) on their [Ca\(^{2+}\)]\(_c\) increase: N-linolenoyl-Gln, N-(15,16-epoxyoctadeca-9,12-dienoyl)-l-Gln, (15, 16-epoxy-Gln), 17-hydroxylinolenoyl-l-Gln (volicitin). C, Effect of the detergent SDS (25 \(\mu\)g mL\(^{-1}\)) on the [Ca\(^{2+}\)]\(_c\) increase in soybean cell cultures.

herbivore Manduca sexta, feeding elicits a jasmonate burst, a large transcriptional reorganization of the plant host and, after hours, a systemic release of terpenoids (Halitschke et al., 2001, 2003). Principally the same sequence is passed through in the interaction between Lima bean and spider mites (Arimura et al., 2000a, 2000b, 2002), and in the interaction of maize (Zea mays) plants with the beet armyworm (Spodoptera exigua; Schmelz et al., 2003; for review, see Gatehouse, 2002).

The results of the present work add novel facets to the previously known sequence and demonstrates that herbivore attack onto a Lima bean leaf is associated with (1) a strong \(V_m\) depolarization at the bite zone causing a wave of \(V_m\) depolarization spreading throughout the entire attacked leaf; and (2) a consistent influx of Ca\(^{2+}\) at the very edge of the bite, which is halved by application of the Ca\(^{2+}\) channel blocker Verapamil. Although none of the amphiphilic N-acyl Glns had a visible impact on the influx of Ca\(^{2+}\) when monitored by fluorescence microscopy in the presence of Fluo-3 AM, the more sensitive assay with the soybean suspension cultures expressing aequorin revealed that these compounds, at least to a certain extent, may be also involved in the Ca\(^{2+}\)-signaling. The quantitative study of the dose dependence effects of N-acyl Gln conjugates upon Ca\(^{2+}\)-influx revealed (1) a concentration-dependent influx of Ca\(^{2+}\); (2) a configuration-independent effect concerning the stereochemistry of the amino acid (Gln); (3) a slightly reduced influx of Ca\(^{2+}\); after modification of the fatty acid backbone by functionalization with oxygen; and (4) a comparable effect with the detergent SDS.

In general, \(V_m\) variations depend on unbalanced ion distribution across the plasma membrane and depolarization occurs when cations (such as K\(^{+}\) and Ca\(^{2+}\)) are allowed to enter the cell or upon anion efflux. On the other hand, hyperpolarization mainly depends on the activity of the plasma membrane H\(^{+}\)-ATPase or when inward anion channels (or outward cation channels) are opened. The primary candidate for intercellular signaling in higher plants is the stimulus-induced change in \(V_m\) (Shvetsova et al., 2001). According to Volkov and coworkers (Volkov, 2000; Shvetsova et al., 2001, and references cited therein) insects induce electrical signals that can influence both biophysical and biochemical processes in remote tissues. Moreover, excitation waves transmit information from one part of the plant to another with a speed of propagation of the action potential that in soybean can reach 40 m s\(^{-1}\) (Shvetsova et al., 2001).

Since ion fluxes through channels directly influence \(V_m\), it seems reasonable to assume that molecules able to act on channel activity might be considered as important factors inducing electrical signals. Among the various channels, calcium channels are predominantly involved in cell signaling and the specificity of the cytosolic calcium concentration signal in triggering a response depends on amplitude, temporal, and spatial changes (White, 2000). In fact, the longer-lasting influxes are expected to penetrate farther into the cytoplasm and therefore encounter more centrally located Ca\(^{2+}\)-dependent enzymes (Trewavas, 2000). Recent studies have raised the challenging idea that depolarization-activated calcium channels can be a sensor for stimuli (such as touch) and might be an exclusive signaling pathway element (Miedema et al., 2001).

Oral secretions and some of its components such as the fatty acid-amino acid conjugates of, for example, Manduca sexta have been shown to be necessary and sufficient to elicit a set of herbivore-specific responses in tobacco (Halitschke et al., 2003). Our results demonstrate that regurgitants and N-acyl-amino acid conjugates interact with the plasma membrane and alter \(V_m\) R from Lima bean reared larvae altered \(V_m\) in a concentration-independent fashion and its effect is clearly different from that observed in \(V_m\) studies with the individual compounds. Especially, the nonlinear response of \(V_m\) to the concentration of R and R-factors remains to be clarified. Possibly the effects are related to different modes of membrane \(V_m\) depolarization by either micellar transport of ions or pore-formations by the conjugates and other components of R (Abramson and Shamoo, 1979). The most common fatty acid conjugate, namely N-linolenoyl-Gln, exhibits \(V_m\) depolarization up to a concentration of 200 \(\mu\)g mL\(^{-1}\), representing the natural concentration in lepidopteran
larvae R (Pohnert et al., 1999a) and this reflects the magnitude of \( V_m \) depolarization observed in R. A typical minor component of lepidopteran R is \( \text{N-palmitoleoyl-Gln} \). At low concentration (25 \( \mu \text{g mL}^{-1} \)), this compound shows \( V_m \) depolarization comparable to \( \text{N-linolenoyl-Gln} \) and \( \text{N-acryloyl-Gln} \). Application of the fatty acid or amino acid components of the conjugates shows virtually no effect for linolenic acid but a clear \( V_m \) depolarization for Gln. The latter effect could play a role during larval feeding after enzymatic cleavage of the conjugates and may rely on transport processes (e.g., symport) of the amino acid (Delrot et al., 2001) and/or interaction of free Gln with receptors. However, as yet, nothing is known about the stability of \( \text{N-acryloyl amino acids} \) in the plant cells.

The time-course and distance-dependence spreading of the \( V_m \) depolarization upon herbivore attack in intact leaves (Fig. 2) is probably associated with a molecule able to disperse within tissues at a relatively high speed. Preliminary results perfusing leaves with \( \text{H}_2\text{O}_2 \) and Ethephon (the ethylene releasing agent) indicate a \( V_m \) depolarizing effect of these molecules (unpublished data).

The typical response pattern to different \( \text{Ca}^{2+} \) concentrations lie in the ability of cells to generate specific cytosolic \( \text{Ca}^{2+} \) concentration signatures. They may be unique, in terms of spatio-temporal characteristics and in response to an individual stimulus (McAinsh and Hetherington, 1998). In the bite zone, there is a dramatic \( \text{Ca}^{2+} \) influx limited to few cell layers (Figs. 6A, 7E1, and 7F1). Usually stimulus-induced increases of \( \text{Ca}^{2+} \) concentration occur in the form of oscillations or in the form of spikes (McAinsh and Hetherington, 1998). In the case of larvae feeding on Lima bean leaves a spike is observed and depends on \( \text{Ca}^{2+} \) channel activity, since the response can be reduced by Verapamil. The involvement of \( \text{N-acryloyl-Gln} \) in \( \text{Ca}^{2+} \) influx is independently confirmed with the test system of aequorin-transformed soybean cells. As previously shown for the established elicitors cryptogenin, oligogalacturonides (Lecourieux et al., 2002), and \( \beta-(1,3)-(1,6)\)-glucans (Mithofer et al., 1999), the \( \text{Ca}^{2+} \) influx increases with increasing \( \text{N-linolenoyl-Gln} \) concentration. Moreover, in our assay, no difference is found between \( \text{D-} \) and \( \text{L-amino acid building blocks of N-linolenoyl conjugate} \), and, thus, the effect of induced \( \text{Ca}^{2+} \) influx is, in the first instance, linked to the overall physico-chemical properties of the amphiphilic compounds, as confirmed by the action of SDS. It should be noted, however, that only the conjugate with \( \text{L-Gln} \) has been shown to induce specific defense responses in maize (Alborn et al., 1997), demonstrating that, besides triggering the \( \text{Ca}^{2+} \) influx, additional interactions between \( \text{N-acryloyl-Gln} \) and the plant are involved in the elicitation process of maize plants. Overall, our findings are in agreement with previous work demonstrating that in Lima bean the signaling pathway(s) mediating expression of defense genes in the receiver cells requires the calcium influx into the cells (Arimura et al., 2000a).

Signals induced by herbivore attack rapidly spread over the leaf leading to a strong \( \text{Ca}^{2+} \)-dependent \( V_m \) depolarization in the bite zone followed by a transient \( V_m \) hyperpolarization in the close vicinity and a constant depolarization in distances greater than 6 to 7 mm. At the long distance (6–7 cm) the overall process takes not more than 5 to 6 min which requires for signal molecules traveling with the same speed (approximately 1 cm min\(^{-1}\)).

Another interesting target is the analysis of the early events in the interaction of volatiles (including ethylene, \( \text{H}_2\text{O}_2 \), and NO) emitted from wounded plants and/or perceived by neighboring healthy plants. Preliminary results already indicate compound-specific variations in \( V_m \). Studies are under way and will be reported soon.

### MATERIALS AND METHODS

#### Plant Material

Feeding experiments were carried out using the Lima bean *Phaseolus lunatus* (cv Ferry Morse var Jackson Wonder Bush). Individual plants were grown from seed in a plastic pot with sterilized potting soil at 23°C and 60% humidity using daylight fluorescent tubes at approximately 270 \( \mu\text{E m}^{-2} \text{s}^{-1} \) with a photophase of 16 h. Experiments were conducted with 12- to 16-d-old seedlings showing two fully developed primary leaves, which were found to be the most responsive leaves.

#### Animal Material

Larvae of *Spodoptera littoralis* (Boisd.; *Lepidoptera, Noctuidae*) were reared on petri dishes at long photoperiod (14–16 h photophase) and 22°C to 24°C. They were fed an artificial diet consisting of 300 g L\(^{-1}\) agar, 400 g L\(^{-1}\) bean flour, 3 g sodium ascorbate, 3 g ethyl \( \text{p-hydroxybenzoate} \), and 1 g formaldehyde (Bergomaz and Boppré, 1986). Small cubes of the diet were placed into rearing dishes on pieces of aluminum foil. Alternatively, larvae were fed with Lima bean leaves. The dishes were lined with filter paper to reduce humidity and sawdust was provided for pupation when the larvae terminated feeding. Adults were allowed to emerge in glass containers supplied with water and honey solution. Eggs were deposited on strips of filter paper.

\( V_m \)

\( V_m \) was determined in leaf segments. The \( V_m \) was determined with glass micropipettes with a tip resistance of 4 to 10 MΩ and filled with 0.5 M KCl. Micropipettes were used as micro-salt bridges to Ag/AgCl electrodes obtained with a Narishighe PE-21 puller and inserted vertically in the tissue by means of a micromanipulator (Maffei et al., 2001). Leaf segments were always equilibrated for 60 to 120 min in 5 mM MES-NaOH (pH 6.0). Perfusion of solutions was granted by a multichannel Ismatec Reglo peristaltic pump (flow rate 1 mL min\(^{-1}\)). \( V_m \) variations were recorded both on a pen recorder and through a digital port of a PC using a data logger. Measurements were performed at increasing distances from the leaf wounded caused by herbivore feeding and the data were plotted with respect to controls. Measurements were also performed after perfusion with the compounds listed below as well as during larvae feeding on intact plants connected to the apparatus.

All chemicals were dissolved in 1% methanol, which was present in the control solutions, and perfused in a 50 mM MES buffered solution (pH 6.0) containing 0.5 mM calcium sulfate and 2.5 mM 3-(3,4-dichlorophenyl)-1,1-dimethylurea, used to poison photosynthetic electron transfer. After a period of \( V_m \) stabilization, saturation of the well where leaf tissues have been placed occurred in 7 min, after which perfusion was carried out for a variable time.
(until stabilization of the $V_m$), washing of the well was done by perfusing with fresh buffer. Saturation with fresh buffer took 20 to 25 min and then the solution was allowed to perfuse until $V_m$ reached a constant value.

**Oral Secretions and Regurgitant Collection**

Five-day-old larvae (approximately 2–3 cm long) were grown on artificial diet and reared on Lima bean leaves for 24 h prior to collection of R. Oral secretions were collected into glass capillaries connected to an evacuated sterile vial (peristaltic pump) by gently squeezing the larva with a forceps behind the head which caused immediate regurgitation. Secretions were stored at (~20°C) until perfusion.

**Intracellular Calcium Variation Determination**

Fluo-3 AM (acetoxy-methyl ester of Fluo-3, more permeant for cells) purchased in vials containing the molecule as a stock solution in dimethyl sulfoxide (Fluka, Milwaukee, WI), was diluted in 50 mM MES buffer, pH 6.0, with the addition of 0.5 mM calcium sulfate, 2.5 mM 3-(3,4-dichlorophenyl)-1,1-dimethylurea to the concentration of 5 mM. This resulting solution was used for an initial treatment of Lima bean leaves not separated from the plant; the leaf was gently fixed over a glass slide, and a drop (about 20 µL) of 5 mM Fluo-3 AM solution was applied and covered with another glass slide. Thirty minutes after treatment with Fluo-3 AM, the leaf was fixed on an Olympus (Tokyo) FV1000 confocal scanning laser microscope stationary without separating the leaf from the plant. Measurements were taken in intact leaves, with leaves wounded mechanically and after herbivore feeding, both in the presence and absence of exogenous calcium. In addition the leaves were perfused with undiluted R and N-linolenoyl-Gln (at 100 µg ml$^{-1}$), as well as with the calcium channel blocker Verapamil (100 µM; White, 2000). The microscope is operated with a Krypton/Argon laser at 488 nm and 568 nm wavelengths; the first wavelength excites the Fluo-3 dye, resulting in emission of green light and the second mostly excites chloroplasts, which emit red fluorescence. Images generated by the FluoView software were analyzed using the public domain NIH Image program (developed at the United States National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image).

**Aequorin Dependent Luminescence Measurements**

Transgenic soybean 6.6.12 cell lines carrying the stably integrated plasmid pGNAequor/neo2 and expressing apoaequorin (Mithöfer et al., 1999) were used to reconstitute aequorin in vivo with 10 µm synthetic coelenterazine (Calbiochem, Bad Soden, Germany) on a shaker (125 rpm) in the dark for 24 h. The Ca$^{2+}$-specific luminescence (470 nm) was measured in a final volume of 200 µL using a digital luminometer (Bio-Orbit 1250, Turku, Finland) as described (Mithöfer et al., 1999; Mithöfer and Mazars, 2002). Treatments with various compounds were performed by adding 1 to 10 µL of different concentrations of aqueous stock solutions to the cell suspension culture. Mixing time for the addition of any compound was 5 to 7 s. In each experiment, the concentration of reconstituted aequorin was not limiting under any of the experimental conditions, with a maximal consumption not exceeding 10%. The residual aequorin was completely discharged by adding 200 µL of 20% ethanol containing 2 mM CaCl$_2$ (final concentration 10% and 1 m, respectively). The luminescence was used to estimate the total amount of aequorin present in various experiments in order to determine the rate of aequorin consumption for the calculation of the cytosolic Ca$^{2+}$ concentrations according to Allen et al. (1977).

**Chemicals Used for Perfusion**

Natural volicitin, 17S-(17-hydroxylinolenoyl)-L-Gln was synthesized as described (Pohnert et al., 1999b). N-linolenoyl-γ-Gln, N-linolenoyl-γ-Gln, the corresponding Glu N-palmitoleoyl-γ-Gln were available according to the protocol of Pohnert et al. (1999a). N-(15,16-epoxy-linolenoyl)-Gln and N-(15,16-dihydroxylinolenoyl)-Gln were synthesized as described by Spiteller and Boland (2003a). Linolenic acid, SDS, and γ-Gln were purchased from Sigma-Aldrich (St. Louis).

**Statistics**

At least five repetitions were used for the statistical treatment of the data. More than five repetitions contributed to the mean values given in Figures 3, 4A to C, and 5. The data are expressed as mean values; metric bars indicate the s.e. To evaluate the difference significance of the control and the treatments at the given concentrations, variance analysis (ANOVA) was performed for all data using the Tukey test.

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