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Cellular responses to naphthoquinones: juglone as a case study

M. Rudnicka · M. Polak · W. Karcz

Abstract The effects of juglone (JG) on the endogenous growth, growth in the presence of either indoleacetic acid (IAA) or fusicoccin (FC) and on proton extrusion were studied in maize coleoptile segments. In addition, membrane potential changes were also determined at chosen JG concentrations. It was found that JG, when added to the incubation medium, inhibited endogenous growth as well as growth in the presence of either IAA or FC. Simultaneous measurements of growth and external pH indicated that inhibition of either IAA-induced growth or proton extrusion by JG was a linear function of JG concentration. Addition of JG to the control medium caused depolarization of the membrane potential ($E_m$), value of which was dependent on JG concentration and time after its administration. Hyperpolarization of $E_m$ induced by IAA was suppressed in the presence of JG. It was also found that for coleoptile segments initially preincubated with JG, although subsequently removed, addition of IAA was not effective in the stimulation of growth and medium acidification. Taken together, these results suggest that the mechanism by which JG inhibits the IAA-induced growth of maize coleoptile segments involves inhibition of PM H$^+$-ATPase activity.

Keywords Juglone · Zea mays · Coleoptile segments · Growth rate · Proton extrusion · Membrane potential

Introduction

Naturally occurring naphthoquinones, such as juglone (JG) (5-hydroxy-1,4-naphthoquinone), lawson (2-hydroxy-1,4-naphthoquinone), plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone) and others, have been studied for a long time due to their importance in medicinal and biological research (O’Brien 1991; Jose 2002; Dayan and Duke 2009). JG is an amber-colored compound isolated from leaves, roots, husks and bark of plants of the Juglandaceae family, particularly black walnut (Juglans nigra). JG and related quinones are referred to as allelochemicals. In agreement with the definition recently proposed by Dayan and Duke (2009), the word allelochemical means a phytotoxin that may negatively influence vegetation in the vicinity of the producing plant. The allelopathic effect of JG on plants is generally toxic, although in some cases it may be also beneficial (Rizvi and Rizvi 1992; Chobot and Hadacek 2009). The toxic effect of JG has been intensively studied in maize (Zea mays L.) and soybean (Glycine max L. Merr). It has been shown in these studies that JG may inhibit the growth of both shoots and roots (Jose and Gillespie 1998; Hejl and Koster 2004; Böhm et al. 2006; Sytykiewicz 2011), photosynthesis (Hejl et al. 1993; Jose and Gillespie 1998), respiration (Jose and Gillespie 1998; Hejl and Koster 2004), transpiration (Jose and Gillespie 1998), and stomatal conductance (Jose and Gillespie 1998). In spite of the large number of papers published on the biological activity of JG (for review see Jose 2002; Dayan and Duke 2009), little is...
known about the mechanism of its toxic effect on plant cells growth.

The main objective of the present study was to determine the effect of JG on IAA-induced growth in maize coleoptile segments. In agreement with the so-called “acid growth hypothesis”, auxin causes acidification of cell wall via stimulation of the plasma membrane H⁺-ATPase that pumps protons into the cell wall and lowers its pH (Rayle and Cleland 1970, 1992; Hager et al. 1971, 1991). In turn, a lower pH either directly lowers the yield threshold of the wall or optimizes the activity of cell wall-localized proteins that loosen the wall (for review see Hager 2003). Both processes provide favorable conditions for cell elongation. Activation of the proton pump by auxin also causes hyperpolarization of the membrane potential and activation of voltage-dependent, inwardly rectifying K⁺ channels, activity of which contributes to water uptake necessary for cell expansion (Cleland et al. 1977; Keller and Van Volkenburgh 1996; Philippar et al. 1999; Becker and Hedrich 2002; Karcz and Burdach 2002, 2007).

It should be added that segments excised from coleoptiles of grasses represent a classical model system for studies on the elongation growth of plant cells. In this system the number of cells is constant and organ grows by elongation only. Moreover, most of crucial evidence on the mechanisms of auxin action in plant cell growth was obtained from maize coleoptile segments. To the best of our knowledge, no research has been reported on the effects of JG on IAA-induced growth in coleoptile cells.

Materials and methods

Plant material

Seeds of maize (Z. mays L. cv. KOKA) were soaked in tap water for 2 h, sown on wet lignin in plastic boxes, and placed in a growth chamber (Type MIR—533, Sanyo Electric Co., Japan) at 27 ± 1 °C. The experiments were performed with 10 mm-long coleoptile segments cut from 96 h old etiolated maize seedlings. Intact coleoptile segments, with the first leaves removed, were excised 3 mm below the tip and collected in an incubation medium of the following composition (control medium): 1 mM KCl, 0.1 mM NaCl, 0.1 mM CaCl₂, initial pH 5.7–6.0.

Chemicals

Juglone (5-hydroxy-1,4-naphthoquinone) was obtained from Sigma Aldrich Inc. (St. Louis, MO, USA). The tested concentrations of JG were prepared by dissolving the allelochemical in distilled water. A 10⁻³ M stock solution is at the limit of JG water solubility (Neave and Dawson 1989). Indole-3-acetic acid (IAA) (Serva, Heidelberg, Germany) was used as a potassium salt, since it could be rapidly dissolved in water. IAA was used at 100 μM. This concentration is optimal for parameters measured for over 10 h in our elongation and pH-measuring apparatus (Polak et al. 2012). For comparison, IAA at 10 μM is optimal for both parameters at the same experimental conditions (e.g. number of coleoptiles per medium volume and composition of the incubation medium), however in short-term recordings (Karcz et al. 1990). Fusicoccin (FC) (Sigma, USA) was dissolved in ethanol and added to the incubation medium at a final concentration of 1 μM. The maximal ethanol concentration of 0.2 % did not affect the growth of coleoptile segments (data not shown).

Growth and pH measurements

Growth experiments with coleoptile segments were carried out in an apparatus that allowed simultaneous measurements of elongation growth and pH of the incubation medium from the same tissue sample (Polak et al. 2012). A somewhat similar procedure for measuring elongation growth of maize coleoptile segments and pH changes of their incubation medium was described previously (Karcz et al. 1990; Karcz and Burdach 2002). Briefly, 60 coleoptile segments were arranged vertically in the apparatus, in three narrow glass pipettes (20 segments in each), connected by means of a silicon hose (Polak et al. 2012, Fig. 1). High-resolution measurements of growth rate were performed with an angular position transducer (TWK Electronic, Düsseldorf, Germany), what resulted in a precise record of the growth kinetics. Coleoptile segments were incubated in an intensively aerated medium. Volume of the incubation medium in the elongation and pH-measuring apparatus amounted to 18 ml (0.3 ml segment⁻¹). The incubation medium also flowed through the lumen of coleoptile cylinders. This feature permitted experimental solutions to be in direct contact with the interior of segments, what significantly enhanced both elongation growth of coleoptile segments and proton extrusion (Karcz et al. 1995). This experimental set-up enabled avoidance of coleoptile abrasion (Dreyer et al. 1981) that inhibits (c. 30 %) elongation growth (Lüthen et al. 1990; Karcz et al. 1995). Medium circulation was driven by a peristaltic pump (1B-05A; Zalimp, Poland). Extension growth of a stack of 20 segments and pH of the incubation medium were sampled every 3 min by a multi-functional computer meter (CX-771; Elmetron, Poland). pH measurements were performed with a pH electrode (OSH 10-10; Metron, Poland). Temperature of all solutions in the elongation and pH-measuring system was thermostatically controlled at a level of 25 ± 0.5 °C. All manipulations and growth measurements were carried out under dim green light.
Electrophysiology

Electrophysiological experiments were performed on coleoptile segments prepared in the same manner as for growth experiments. A standard electrophysiological technique was used for membrane potential measurements, as previously described (Karcz and Burdach 2002; Kurtyka et al. 2011). Briefly, membrane potential (Em) was measured by recording the voltage between a 3 M KCl-filled glass micropipette inserted into the parenchymal cells and a reference electrode in the bathing medium of the same composition as used in growth experiments. For electrophysiological experiments, the segments were preincubated for 1 h in an intensively aerated bathing medium, whereupon juglone was added. Inset on the right side shows the total elongation growth, calculated as the sum of extensions measured at 3-min intervals over 10 h, while inset on the left shows the effect of juglone on the pH of medium (with maize coleoptile segments), measured simultaneously with growth. The data presented are means of at least seven independent experiments. Bars indicate means ± SEs.

Statistical analysis

Data were analyzed with the Statistica computer software for Windows (StatSoft 2008, STATISTICA data analysis software system, version 8.0 http://www.statsoft.com, USA).

Results

Effect of juglone (JG) on the endogenous growth, growth in the presence of IAA, and medium pH measured simultaneously with growth

Addition of JG to the incubation medium inhibited, in a concentration-dependent manner, the endogenous growth (growth in a medium without growth substances) of maize coleoptile segments (Fig. 1). In the presence of 10 μM JG, stimulation of endogenous growth was observed within the first 5 h. Measurements of the medium pH, taken simultaneously with the growth of maize coleoptile segments (Fig. 1, inset on the left), indicated that at concentrations of 75 and 100 μM JG caused alkalinization of the incubation medium, whereas at 10 μM stimulated medium acidification, as compared to the control. At 50 μM, after an initial transient alkalinization of the incubation medium, JG induced a slow decrease of medium pH.

As can be seen in Fig. 2, auxin added to the control medium (after 2 h of preincubation) with maize coleoptile segments induced their strong growth rate, kinetics of which could be divided into two phases (biphasic reaction). The first phase, very rapid, was followed by a long-lasting
one which began about 30 min after auxin addition. Administration of JG (after 1 h incubation of coleoptile segments in a control medium) inhibited the growth of coleoptile segments. JG at 10 μM diminished the second (long-lasting) phase of IAA-induced growth, while at concentrations of 50 and 75 μM proportionally reduced both phases (Fig. 2). In turn, JG at the highest concentration (100 μM) abolished the first and second phase of IAA-induced growth. On the basis of growth rate responses shown in Fig. 2, the total elongation growth over 10 h, calculated as the sum of extensions measured at 3-min intervals over 10 h. All curves are means of at least 9 independent experiments. Bars indicate means ± SEs.

Figure 4 shows interdependencies between the effects of JG on the IAA-induced growth and medium pH measured simultaneously with growth. These data indicate that inhibition of either IAA-induced growth or proton extrusion by JG is a linear function of JG concentration. The linear regression fit of both magnitudes delivers a similar (negative) slope. Taking the above into account, it may be deduced that proton extrusion and elongation growth are strongly correlated with JG concentration.

For comparison, the effect of JG at 100 μM on FC-induced growth and medium pH has also been shown. This fungal toxin is known to enhance H⁺-ATPase activity, through phosphorylation of the penultimate Thr, as well as to induce elongation growth (Marrè 1979; Kinoshita and Shimazaki 2001). FC added to incubation medium in the same way as IAA, at a final concentration of 1 μM, enhanced endogenous growth of maize coleoptile segments to the level comparable with the growth seen in the presence of IAA (Fig. 5). As indicated in Fig. 5, FC added at 2 h was much more effective in acidifying of the medium, as compared to IAA. For FC, 8 h
after its addition, pH of the incubation medium dropped to approximately 3.6, whereas for IAA pH attained only 4.8. It has also been found that FC is much more effective than IAA in stimulating the elongation growth of maize coleoptile segments which were previously incubated in the presence of JG. For example, JG applied to the medium (after 1 h of preincubation) at a concentration of 100 μM decreased the growth of coleoptile segments incubated in the presence of
IAA by 90%, while in the presence of FC only by 20%. pH of the incubation medium recorded after 10 h in the presence of JG (100 μM) and FC was approximately by 0.3 pH unit higher than for FC alone (Fig. 5). However, in the case of IAA, JG at 100 μM abolished IAA-induced proton extrusion, causing alkalinization of the incubation medium.
IAA-induced hyperpolarization of $E_m$ was observed. Interchanging medium changed for JG with IAA, a suppression of time 0, the control medium was changed for a new one, of the same salt composition, containing JG, IAA or JG with IAA.

Effect of juglone on the membrane potential ($E_m$)

Addition of JG to the control medium caused depolarization of the $E_m$ value, which was dependent on JG concentration and time after its administration (Table 1). For example, treatment of parenchymal cells from maize coleoptile segments with 75 or 100 µM JG caused depolarization of the $E_m$ by 10 and 55 mV, respectively. In turn, addition of IAA to the control medium caused, after over 1 h, hyperpolarization of the $E_m$ by 14.5 mV (from $-111.7 \pm 5.3$ to $-126.2 \pm 5.2$ mV). For coleoptile segments initially preincubated with JG at 75 µM ($E_m = -99.7 \pm 4.1$ mV), for 1 h, and subsequently kept in a bathing medium changed for JG with IAA, a suppression of IAA-induced hyperpolarization of $E_m$ was observed. Interestingly, JG at 75 µM not only suppressed IAA-induced hyperpolarization of the $E_m$ but also caused an additional membrane depolarization (Table 1).

Discussion

Despite the availability of extensive research on JG toxicity, our knowledge on the effect of this allelochemical on plant growth is still limited. The main goal of this work was to determine the mechanisms by which JG inhibits IAA-induced growth of maize coleoptile segments. In the study, we selected three response parameters concerning auxin action: elongation growth, proton extrusion and membrane potential. Interdependences between these parameters provided basis for the “acid growth hypothesis” of auxin action.

Data presented in this paper, showing that IAA causes (1) acceleration of elongation growth, as compared to growth in control (Fig. 2), (2) enhancement of proton extrusion, as compared to IAA-free medium (Fig. 3), and (3) hyperpolarization of the membrane potential (Table 1), are in good agreement with results obtained by other investigators (Cleland et al. 1977; Kutschera and Schopfer 1985; Felle et al. 1991; Lüthen et al. 1990; Rücke et al. 1993; Keller and Van Volkenburgh 1996; Karcz and Burdach 2002, 2007; Kurytka et al. 2011). There is no doubt that plasma membrane hyperpolarization observed in the presence of IAA is a consequence of stimulated proton extrusion through H$^+$-ATPase (Cleland et al. 1977; Lohse and Hedrich 1992; Rücke et al. 1993; Hedrich et al. 1995).

Simultaneous measurements of elongation growth and medium pH showed that in maize coleoptile segments JG inhibited both IAA-induced growth (Fig. 2) and proton extrusion (Fig. 3) in a concentration-dependent manner. The linear regression fit of both magnitudes delivered a similar (negative) slope, suggesting that growth and proton concentration are strongly correlated with JG concentration (Fig. 4). This finding is in good agreement with the “acid growth hypothesis” of auxin action (for review see Hager 2003) and clearly indicates that changes in IAA-induced growth of maize coleoptile cells observed in the presence of JG are mediated via PM H$^+$-ATPase activity. This suggestion is also supported by two facts: first, that the FC-induced growth and proton extrusion (Fig. 5), attributed to PM H$^+$-ATPase activity (Marrè 1979), were also diminished in the presence of JG, and second that JG depolarized and abolished the IAA-induced hyperpolarization of $E_m$ (Table 1). In contrast to IAA, FC was much more effective in stimulating both the growth and medium acidification of maize coleoptile segments treated with JG. This observation probably results from the fact that IAA and FC differ in their signal transduction pathway (Hager 2003). At least in maize coleoptile cells, auxin-induced medium acidification is

### Table 1 $E_m$ changes in parenchymal coleoptile cells with addition of juglone (JG), indole-3-acetic acid (IAA) and JG together with IAA. At time 0, the control medium was changed for a new one, of the same salt composition, containing JG, IAA or JG with IAA.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>$E_m$ (mV)</th>
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<tbody>
<tr>
<td></td>
<td>$E_m$ after stabilization in control medium (time 0)</td>
</tr>
<tr>
<td>50 µM JG</td>
<td>$-107.9 \pm 4.3$</td>
</tr>
<tr>
<td>75 µM JG</td>
<td>$-108.6 \pm 5.7$</td>
</tr>
<tr>
<td>100 µM JG</td>
<td>$-110.6 \pm 4.8$</td>
</tr>
<tr>
<td>100 µM IAA</td>
<td>$-111.7 \pm 5.3$</td>
</tr>
<tr>
<td>75 µM JG +IAA</td>
<td>$-99.7 \pm 4.1$</td>
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In the case of adding JG together with IAA, coleoptile segments were first preincubated for over 1 h in the presence of juglone, whereupon $E_m$ was measured. Data are means of at least eight independent experiments. Error indicates ±SE.
mediated by an increased activity and/or amount of PM H+-ATPase, while FC stimulates only proton extrusion by the increased PM H+-ATPase activity (Hager et al. 1991; Frias et al. 1996; Philippar et al. 1999). Interestingly, it has been only recently shown (Takahashi et al. 2012) that application of IAA to endogenous auxin-depleted hypocotyl sections of Arabidopsis seedlings induced phosphorylation of the penultimate threonine of H+-ATPase and increased H+-ATPase activity without altering the amount of enzyme. In addition, these authors also showed that an auxin antagonist specific for the nuclear receptor TIR1/AFBs had no effect on the IAA-induced H+-ATPase phosphorylation.

What would be the mechanism by which JG inhibits IAA-induced growth of maize coleoptile cells? Evidently, inhibition of PM H+-ATPase activity by JG plays a key role in this phenomenon. In addition, our data, showing that JG depolarized the E_m and abolished the IAA-induced plasma membrane hyperpolarization, might suggest that voltage-dependent, inwardly rectifying K+ channels play a role in growth regulation of maize coleoptile cells incubated with JG. Interestingly, in patch-clamp experiments, Varga et al. (1996) observed that voltage-gated K+ channels in human lymphocytes were sensitive to externally applied JG with a half blocking concentration of approximately 57 μM. Blocking of K+ channels by JG was in concert with its depolarizing effect on human lymphocytes. Taking into account the above remarks, Varga et al. (1996) suggested that the reduced K+ channel activity reported in the presence of JG may also be responsible for its cell growth inhibiting effect. It should be also added that voltage-gated K+ channels involved in IAA-induced growth of maize coleoptile cells and K+ channels identified in human lymphocytes belong to the same family of voltage-gated (Shaker-like) K+ channels (reviewed in Dreyer and Uozumi 2011).

Considering the mechanisms by which naphthoquinones may inhibit the PM H+-ATPase activity, two major types of reaction should be taken into account (Rossi et al. 1986; Brunmark and Cadenas 1989; Öllinger and Brunmark 1991). The first is a direct interaction between the enzyme and quinone which leads to covalent modification of protein thiols and generation of thioethers (arylation process). The next mechanism is the capacity of quinone to produce reactive oxygen species (ROS), via redox cycling, and interaction with nucleophilic biomolecules, such as protein thiols or glutathione. It should be added that ROS were identified as an activator of Ca2+ influx channels which could be responsible for membrane potential depolarization (Schroeder et al. 2001). Interestingly, both mechanisms are associated with inhibition of jack bean urease activity by JG (Kot et al. 2010). Another possibility is that JG, being a strong electron acceptor, may inhibit plasma membrane redox systems, which, in plant cells, are involved in elongation growth, proton release and plasma membrane depolarization (for review see Lüthje et al. 1997). Moreover, it was also shown that auxin and FC modify plasma membrane redox activity (Lüthén and Böttger 1993; Le-Kacz and Karcz 2006).

The inhibitory effect of JG on the growth of maize coleoptile segments incubated in the presence of IAA may also result from the reduced rate of degradation of AUX/IAA proteins (family of transcriptional regulators) due to their inhibited interaction with the SCF–TIR1 complex. Isomerization of prolines in domain II AUX/IAA, essential for the proper recognition of AUX/IAA by the SCF–TIR1 complex, is carried out by an enzyme of the PPIs family, parvulin. JG is known as a specific inhibitor of parvulin, interacting only with the PPIs subfamily (Hennig et al. 1998; Dharmasiri et al. 2003; Tian et al. 2003; Kepinski and Leyser 2004). Another possible explanation for the inhibitory effect of JG on the growth of maize coleoptile segments is its interaction at the level of gene transcription. JG may have the ability to inhibit RNA polymerase II, I and III within an interaction with thiol groups of cysteines of the conserved regions in all three polymerases (Chao et al. 2001).

Taking the above into account, it may be suggested that the inhibitory effect of JG results from two independent pathways; the first one associated with inhibition of PM H+-ATPase and second linked to system operating via the E3 ubiquitin-ligase complex SCF^{TIR1/AFB}, which includes TIR1/AFBs. These two pathways may reflect the effect of JG on the “fast” and “slow” phase of IAA-induced growth rate in maize coleoptile segments, respectively.

The herein presented results are generally in agreement with data obtained by Hejl and Koster (2004), who showed that JG inhibited H+-ATPase activity in corn and soybean root microsomal fractions. They also found that corn and soybean seedlings grown in a nutrient solution amended with 10, 50 or 100 μM JG (the same concentrations were also used in our experiments) showed a significant decrease in root and shoot dry weights and lengths. Interestingly, Hejl and Koster (2004) also observed that JG diminished the ability of roots to extrude protons and uptake water.

In conclusion, results presented in this article demonstrate that the mechanism by which JG inhibits the IAA-induced growth of maize coleoptile segments involves inhibition of PM H+-ATPase activity. This suggestion is supported by three lines of evidence: (1) inhibition of either IAA-induced growth or proton extrusion by JG is a linear function of JG concentration (Fig. 4); (2) JG added together with IAA suppresses the IAA-induced hyperpolarization of the E_m (Table 1); (3) FC-induced growth and proton extrusion are diminished in the presence of JG (Fig. 5).
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