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Harpin, a hypersensitive response elicitor from *Erwinia amylovora*, regulates ion channel activities in *Arabidopsis thaliana* suspension cells

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Abstract HrpN, the hypersensitive response elicitor from *Erwinia amylovora*, stimulated K^+ outward rectifying currents in *Arabidopsis thaliana* suspension cells. It also decreased anion currents. These data demonstrate the ability of harpin to regulate different plasma membrane ion channels, putative components of signal transduction chains leading to defense responses and programmed cell death. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Harpin; *Erwinia amylovora*; *Arabidopsis thaliana*

1. Introduction

Plants frequently respond to pathogen attack by the 'hypersensitive reaction' (HR), a rapid necrosis at the site of infection that cordons off the pathogen and limits its spread [1,2]. The HR cell death bears similarity to the programmed cell death (PCD) or apoptosis observed in animal cells [2]. Following pathogen recognition, the earliest reactions detectable are the regulation of specific ion channels and the formation of reactive oxygen intermediates [3,4]. Harpin, a bacterial HR elicitor is a heat-stable, glycine-rich protein that was first described in *Erwinia amylovora* [5]. This bacterial pathogen causes fire blight disease of apple, pear and other members of the Rosaceae. Harpin proteins from various bacterial plant pathogens have been characterized [6–10]. In suspension cell cultures from tobacco or *Arabidopsis*, harpin induces early responses such as potassium efflux and the rapid inhibition of ATP synthesis [11], which should induce inhibition of the H^+-ATPase resulting in plasma membrane depolarization [12] and alkalinization of the growth medium [13,14]. An increase in cytosolic calcium [15], the production of active oxygen species [16,17] and the induction of mitogen-activated kinase [18,19] have also been reported. All these events have been discussed as components of signal transduction chains leading to defense response and/or cell death [3,4,20–22]. In this study, we used *Arabidopsis thaliana* suspension cultures to demonstrate the regulation of anion and potassium currents by the *E. amylovora* harpin hrpN.

2. Materials and methods

A. thaliana L. (ecotype Columbia) suspension cells were cultured at 24 ± 2°C, under continuous white light (40 μE m^{-2} s^{-1}) with rotation shaking, in a 1-l round bottom flask containing 350 ml Gamborg culture medium (main ions: 25 mM K^+, 1 mM Ca^{2+}, 25 mM NO_3^-, 2 mM Cl^- [23]). The pH of the culture medium was 5.8. Cells were subcultured weekly by a 10-fold dilution. The experiments were conducted on 4-day-old cultures. The suspension cells were impaled in the culture medium as previously described [24,25]. The microelectrode resistance was 40–50 MΩ when filled with 600 mM KCl. Individual cells were voltage-clamped using an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA, USA) for discontinuous single electrode voltage clamp experiments [26]. Voltage and current were digitized with a personal computer fitted with a Digidata 1320A acquisition board (Axon Instruments). The electrometer was driven by pClamp software (pCLAMP8, Axon Instruments). Experiments were performed at 22 ± 2°C. The $harpin_{ea}$ was prepared and partially purified as described by Pike et al. for electrophysiological studies [12]. Briefly, *Escherichia coli* strain K38 (pGp1-2; pMAB64) containing the cloned *hrp*N gene [27] was grown up to an optical density at 600 nm of 0.4 and T7 RNA polymerase was induced according to Tabor et al. [28]. The cells were harvested, resuspended in 2 ml of phosphate buffer (pH 7, 10 mM), boiled for 10 min and centrifuged. The supernatant was recovered and concentrated using Centricon10 (Amicon). To assess the contribution of *E. coli* protein background to membrane response, a cell free preparation of *E. coli* strain K38 (pGp1-2; pT7-7), containing the pT7-7 vector without the *hrp*N insert, was prepared in parallel to harpin and used as negative control. Protein contents of both preparations were compared on an 8% SDS–polyacrylamide gel and quantified using the method of Bradford [29]. This indicates that harpin represent 75% of the total protein preparation.

3. Results and discussion

The membrane potential and ion current characteristics of the *A. thaliana* suspension cells were similar to those we previously reported in close conditions [24,25]. The membrane potential (V_m) of cells was -40 ± 11 mV (mean ± S.D., $n = 61$). Anion currents were detected in 75% of the cells after negative pulses (Fig. 1A). These currents were due to anion efflux as we previously described [25]. These currents displayed the principal hallmarks (Fig. 1B) of slow type anion channels [30,31]. In addition, time- and voltage-dependent outward rectifying currents (Fig. 2A,B) were activated by depolarizing pulses. These currents were due to K^+ efflux and named KORC for K^+ outward rectifying currents [24].

Harpin induced, in less than 1 min, a decrease in anion currents (Fig. 1A,B). This decrease was dose-dependent for the instantaneous peak anion currents (Fig. 1A–C) but also at steady state as shown on the current–voltage relationships (inset in Fig. 1A). Note that the treatment with the negative control (without harpin) induced a large increase in these

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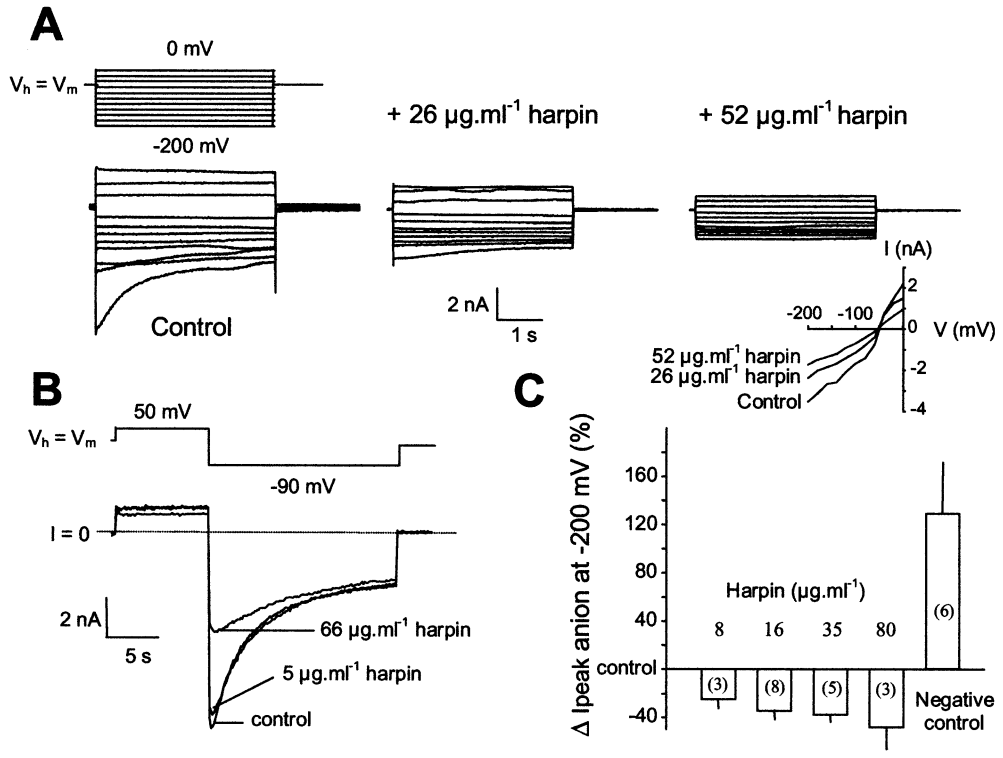

Fig. 1. Effect of harpin on the plasma membrane anion currents of intact *A. thaliana* suspension cells. (A) Anion currents measured in control conditions (in culture medium at pH 5.8, main ions: 25 mM KNO_3, 1 mM $CaCl_2$), 1 min after adding 26 µg ml^{-1} harpin and after the addition of 52 µg ml^{-1} harpin. Voltage pulses ranged from −200 to 0 mV (in 20 mV steps for 4 s). Holding potential was V_m. Corresponding current–voltage curves determined from currents recorded at 3800 ms after deactivation. (B) Superimposition of anion current traces activated by a depolarizing prepulse (50 mV for 10 s) and then by a 20 s hyperpolarizing pulses of −90 mV, with or without harpin. (C) Changes in peak anion current (ΔI) at −200 mV after treatment with various concentrations of harpin or with the negative control (harpin −). The protein content of the negative control (27 µg ml^{-1}) corresponds to the content of protein background present in addition to the 80 µg ml^{-1} of harpin (80 = 75% of the total 107 µg of protein per ml). Same protocol as in A. The numbers in brackets correspond to the number of impaled cells for each treatment. Standard error bars are shown.

anion currents (Fig. 1C) suggesting that the harpin-induced decrease in current may be underestimated. This decrease in anion current induced weak membrane hyperpolarization in some cells: $\Delta V_m = -5.0 \pm 2.8$ mV ($n = 8$). This hyperpolarization was delayed, reaching a steady state value 8 ± 2.8 min after harpin application. The effect of harpin, corresponding to a decrease of anion efflux, was the opposite of that described for various pathogen elicitors, which were shown to increase Cl^- efflux [20,32]. However, the anion current decrease we report here occurs much earlier than the Cl^- efflux increase previously reported [20,32], possibly indicating different roles for these anion fluxes. The decrease in anion currents, through inhibition of cystic fibrosis transmembrane conductance regulator Cl^- channels, induces apoptosis in hepatoblastoma cells [33]. Thus, the early regulation of anion currents we described is likely to be part of the harpin-induced transduction cascade leading to gene expression regulation, as

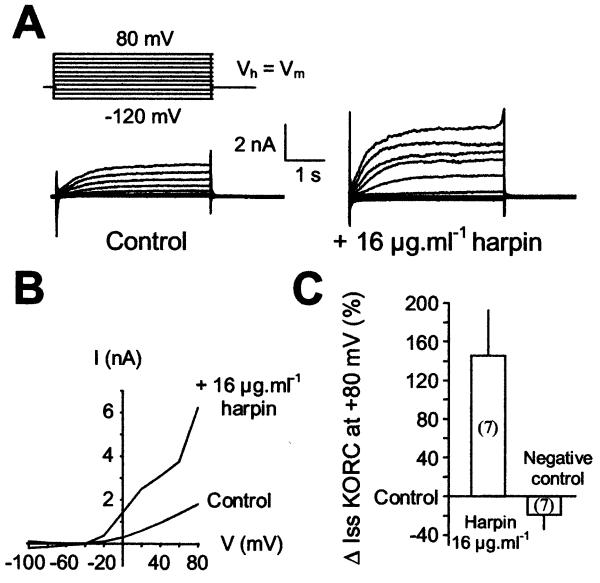

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Fig. 2. Effect of harpin on the time-dependent KORC of intact *A. thaliana* suspension cells. (A) KORC measured in control conditions (in culture medium at pH 5.8, main ions: 25 mM KNO_3, 1 mM $CaCl_2$) and 1 min after adding 16 µg ml^{-1} harpin. (B) Corresponding steady state current–voltage curves. Voltage pulses ranged from −120 to +80 mV (in 20 mV steps for 4 s). Holding potential was V_m. (C) Changes in KORC at steady state (ΔI_{ss}), at 80 mV, after treatment with 16 µg ml^{-1} harpin or with the corresponding negative control (harpin −). The numbers in brackets correspond to the number of impaled cells for each treatment. Standard error bars are shown.

we demonstrated for the ABA response in *A. thaliana* suspension cells [25].

A low dose of harpin increased the K^+ efflux through KORC (Fig. 2A–C) in about 1 min. The negative control induced a weak decrease in KORC (Fig. 2C). The harpin-induced increase in KORC, corresponding to an increase of K^+ efflux through K^+ channels, may be related to the increase in electrolyte leakage, and particularly K^+, in response to harpin treatment previously reported on tobacco suspension cells [12,13]. Studies in animal models indicate that a decrease in intracellular K^+ concentration is a necessary early event in PCD [34,35]. In plants, cell death has been correlated with the leakage of ions from leaf discs [36]. Furthermore, HR-activated protein, which belongs to a novel gene family called HIR [37], appears to play a role in cell death by regulating potassium channels, especially in response to disease [37].

Harpin was recently described to form in vitro a cation selective conducting pore [38]. The in vitro pore-induced current does not exhibit the rectifying properties we observed in vivo for KORC. Furthermore, the harpin pores observed in vitro could not account for the regulation of anion currents we observed in vivo. All these data suggest that insertion of harpin into the membrane induces the regulation of several ion channel activities in vivo. These regulations could be an early step of the harpin-induced hypersensitive cell death reported in tobacco [11] as well as in *A. thaliana* suspension cells [17].

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