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A CFTR chloride channel activator prevents $HrpN_{ea}$-induced cell death in *Arabidopsis thaliana* suspension cells

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Abstract

Erwinia amylovora is a necrogenic bacterium that causes fire blight of the Maloideae subfamily of *Roseacae*, such as apple and pear. It provokes necrosis in aerial parts of susceptible host plants and the typical hypersensitive reaction in non-host plants. The secreted harpin, $HrpN_{ea}$, is able by itself to induce an active cell death in non-host plants. Ion flux modulations were shown to be involved early in such processes but very few data are available on the plasma membrane ion channel activities responsible for the pathogen-induced ion fluxes. We show here that $HrpN_{ea}$ induces cell death in non-host *Arabidopsis thaliana* suspension cells. We further show that two cystic fibrosis transmembrane conductance regulator modulators, glibenclamide and bromotetramisole, can regulate anion channel activities and $HrpN_{ea}$-induced cell death.

Keywords: Anion channel; *Arabidopsis thaliana*; Cell death; CFTR; Harpin

1. Introduction

Erwinia amylovora is a bacterial pathogen that causes fire blight disease of apple, pear and other members of the *Rosaceae*, its host plants. It secretes the $HrpN_{ea}$ harpin, a "hypersensitive response" (HR) elicitor [42]. HR cell death is a response of non-host plant to pathogen attack and consists of a rapid necrosis at the site of infection that cordons off the pathogen and limits its spread [8,15]. There is a growing consensus that HR is similar to animal programmed cell death (PCD) and that ion channel regulation is a necessary event to induce PCD [15,16,21,31,44,45]. In plants, plasma membrane potential and ion flux variations are among the earliest signaling events detectable in response to pathogens and elicitors [30,32,34,45]. However, the underlying ion channel activities have been recorded rarely. Indeed, most of the electrophysiological studies on plant cells are performed with patch-clamp technique applied to protoplasts and it seems that the cell wall removing (protoplasts preparation) alters the cell response capacity to pathogen or elicitor [4,19]. Another approach to analysis of ion channels in intact cells that retains their cell wall is the microelectrode voltage-clamp technique. This technique allows long-term recording of the free running membrane potential and whole cell ion currents, the internal medium remaining physiological (composition non-controlled). We have shown previously, using single microelectrode voltage-clamp (DSEVC), that *Arabidopsis thaliana* suspension cells respond to the fungal elicitor hypaphorine in the same way as root hairs, its natural target [36]. Other studies demonstrated that suspension cultured cells are a powerful system of reduced complexity to analyze the signal transduction pathway induced by pathogens [7,29,43]. Thus, we used *A. thaliana* suspension cells to inves-

Abbreviations: AHAS, acetohydroxyacid synthase; AVD, apoptosis volume decrease; CFTR, cystic fibrosis transmembrane conductance regulator; DMSO, dimethyl sulfoxide; DSEVC, discontinuous single electrode voltage-clamp; HR, hypersensitive response; KORC, K^+ outward rectifying current; MAPK, mitogen activated protein kinase; PCD, programmed cell death; SU, sulfonylurea molecule.

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tigate early signaling events induced by $HrpN_{ea}$. We showed previously that $HrpN_{ea}$ could modulate K^+ and anion currents [10] suggesting indirect effects on the channel proteins. Regulation of anion fluxes was reported in pathogen-induced plant cell death. For example, a large nitrate efflux is necessary to induce cell death in tobacco in response to cryptogein [25,43]. Moreover, anion channel antagonists have been shown to interfere with elicitor or pathogen-induced responses such as Ca^{2+} influx [9], production of active oxygen species [17,35], MAPK activation [26], and phytoalexin synthesis [9,17]. In animal cells, Maeno et al. [27] showed that apoptosis volume decrease (AVD: cell shrinkage) is a major hallmark of PCD. This AVD is due to a strong activation of ion effluxes. In plant cells, the increase of anion effluxes in response to pathogen elicitors [43,45] is consistent with the AVD. However, in *A. thaliana* suspension cells, we observed a decrease in anion current (efflux decrease) in response to $HrpN_{ea}$ [10]. These data did not fit with the observations described above but are closely related to those reported for hepatoblastoma apoptosis [18]. In this last model, apoptosis is induced by the decrease in cystic fibrosis transmembrane conductance regulator (CFTR) anion currents (members of ABC transporter superfamily). In plant, CFTR modulators, were shown to be effective on slow anion channels of *Vicia faba* guard cells [23]. Moreover, Leonhardt et al. [24] showed, using antibodies, that slow anion channels are, or are closely, controlled by a polypeptide exhibiting an epitope shared with the mammalian CFTR. Lastly, AtMRP5, a protein of the ABC transporter superfamily, which has a high similarity to CFTR and which is sensitive to glibenclamide, was suggested to control ion channels [12]. In this study, our aim was to investigate the putative involvement of the anion current decrease in $HrpN_{ea}$-induced cell death on *A. thaliana* suspension cells (non-host plant) by using CFTR modulators, glibenclamide, a sulfonylurea (SU) molecule, as an inhibitor and bromotetramisole as an activator, according to the hypothesis developed on hepatoblastoma [18].

2. Results

$HrpN_{ea}$ at 5 μg ml^{-1} (0.13 μM), a classically used $HrpN_{ea}$ concentration [42,33], increases cell death in comparison with the cells treated with negative control (Table 1). According to our hypothesis we thus tested in our model the effect of glibenclamide and bromotetramisole. In animal systems, the concentrations of glibenclamide tested are highly variable: whole-cell CFTR-Cl^- currents are inhibited at a half-maximal concentration of 20 μM [39], apoptosis is induced at 1 mM [18]. Thus, we tested the effect of different concentrations of glibenclamide on anion currents. The deactivating currents (Fig. 1A), previously characterized as anion currents [3,10,36], are sensitive to glibenclamide in a dose-dependent manner (Fig. 1). The glibenclamide concentration for half-maximum inhibition of anion current observed in our model is about 7 μM (Fig. 1B), of the same order of magnitude as those observed for slow-type anion currents on plant cells [23]. Thus, we tested the effect of 10 μM glibenclamide on cell death. After 24 h, glibenclamide increased cell death, mimicking the $HrpN_{ea}$-induced cell death (Table 1). Although glibenclamide is effective on anion currents in our model (Fig. 1) and in *V. faba* guard cells [23,24], we checked if the glibenclamide-induced cell death might be due to other known glibenclamide or SU effects, i.e. K^+ channel inhibition [22] or herbicidal activity [5,6]. Glibenclamide (10 μM) does not induce a change in K^+ outward rectifying current (KORC) activity of *A. thaliana* suspension cells (ΔI_{KORC} at steady state for a + 80 mV voltage step = 1.3 ± 9%, $n = 6$, data not shown). Yet in plants, the herbicidal activity of SU family acts through AHAS inhibition [5,6]. AHAS catalyses the for-

Table 1
Modulation of $HrpN_{ea}$-induced cell death by anion channel modulators
Increase in cell death after a 24 h treatment with glibenclamide, $HrpN_{ea}$ alone or mixed with bromotetramisole. Variations are given as a percentage with respect to the control level. Data correspond to mean values ± S.D. and n is the number of independent experiments

	5 μg ml^{-1} $HrpN_{ea}$	10 μM Glibenclamide	5 μg ml^{-1} $HrpN_{ea}$ + 5 μM bromotetramisole
Cell death (%)	21 ± 3 ($n = 6$)	26 ± 4 ($n = 3$)	1.3 ± 2.8 ($n = 3$)

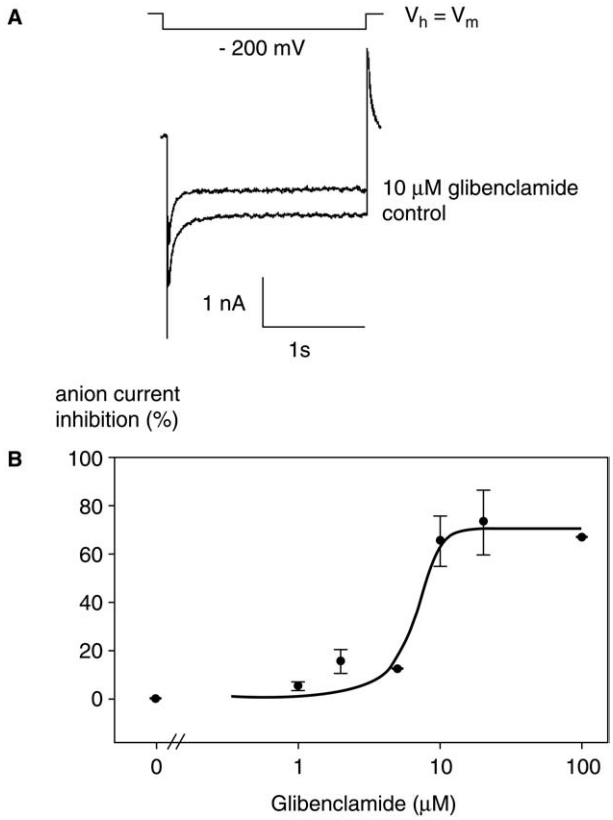

Fig. 1. Effect of glibenclamide on anion currents.
(A) Anion currents measured under control conditions and after adding 10 μM glibenclamide in the culture medium. Voltage pulses were –200 mV. Holding potential was V_m. (B) Dose-dependent decrease of anion currents as a function of glibenclamide concentrations. Data represent the maximal effect. They were obtained from at least three independent experiments and were fitted by a 5-parameter double exponential. The error bar corresponds to one standard error.

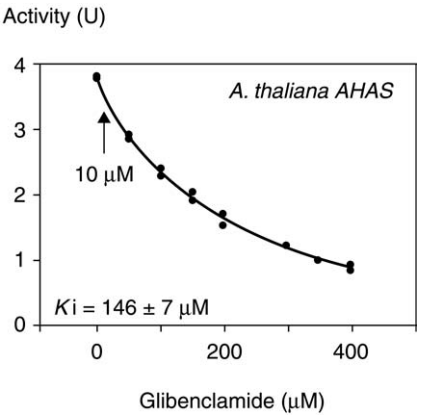

Fig. 2. Effect of glibenclamide on AHAS activity.
Inhibition of *A. thaliana* AHAS activity. Duplicate measurements were made at each inhibitor concentration and the graph shows all values as separate points. From the data shown, the inhibition constant (*K*i) was determined. The arrow indicates the AHAS inhibition at 10 µM.

mation of 2-acetolactate and 2-aceto-2-hydroxybutyrate as the first step in the biosynthesis of the branched-chain amino acids valine, leucine and isoleucine. To check if glibenclamide-induced cell death involves this type of herbicidal effect or acts through ion channel regulation, we have tested whether glibenclamide could inhibit AHAS from *A. thaliana*. The residual AHAS activity at 10 µM glibenclamide, compared to the corresponding control, was greater than 95% for *A. thaliana* AHAS. The inhibition constant (*K*i) calculated for AHAS, 146 ± 7 µM (Fig. 2), is much higher than those of known herbicidal sulfonylureas which have inhibition constants in the 10–100 nM range for *A. thaliana* AHAS [6].

In contrast to glibenclamide, the anion currents down regulated by $HrpN_{ea}$ [10] are stimulated by bromotetramisole in a dose-dependent manner (Fig. 3). The bromotetramisole half-maximum activation concentration we observed for anion current in our model is about 20 µM (Fig. 3B). Bromotetramisole appeared thus as a useful tool to check if the increase of anion efflux could interfere with $HrpN_{ea}$-induced cell death. We tested bromotetramisole at 5 µM a low concentration that is able to increase anion currents. At this concentration, bromotetramisole counteracted the $HrpN_{ea}$-induced cell death (Table 1).

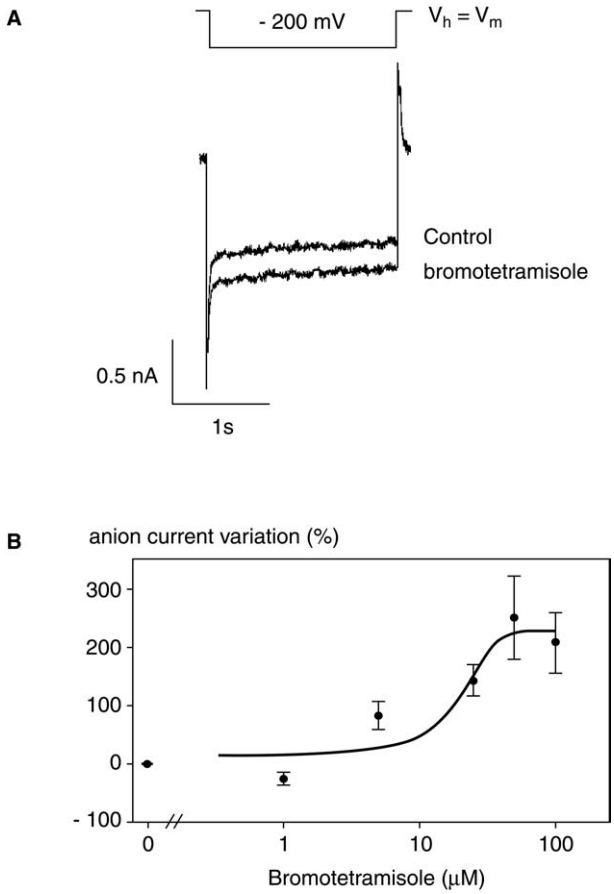

Fig. 3. Effect of bromotetramisole on anion currents.
(A) Anion currents measured under control conditions and after adding 5 µM bromotetramisole in the culture medium. Voltage pulses were –200 mV. Holding potential was V_m. (B) Dose-dependent increase of anion currents as a function of bromotetramisole concentrations. Data represent the maximal effect. They were obtained from at least three experiments and were fitted by a 5-parameter double exponential. The error bar corresponds to one standard error.

3. Discussion

In non-host plants, $HrpN_{ea}$ is known to induce HR cell death [42], symptom of aggression by an avirulent pathogen. We showed that $HrpN_{ea}$ could induce cell death in *A. thaliana* suspension cells, a model non-host plant. The extent of $HrpN_{ea}$-induced cell death was in the same range as observed with another harpin, HrpZ, on *A. thaliana* suspension cells [20]. In addition to cell death, $HrpN_{ea}$ induces a decrease in anion currents (anion effluxes) [10]. This result is not in accordance with previous studies suggesting that anion effluxes are a part of the pathogen or elicitor induced responses [9,17,26,35] necessary to induce cell death [25,43]. However, this decrease fits well with the PCD observed on hepatoblastoma cells by Kim et al. [18]. These authors showed that a decrease of CFTR anion current is a necessary step of PCD development. To test the hypothesis that a decrease in anion current is involved in cell death, we used CFTR modulators. Glibenclamide is believed to bind the CFTR channel allowing its block [46]. Glibenclamide is also a well-known inhibitor of ATP-dependent K^+ channels used as a therapeutic agent to treat type 2 diabetes [28]. If the increase in KORC induced by $HrpN_{ea}$ [10] was involved in cell death, the putative inhibition of KORC by glibenclamide should favor survival of cells. However, in our model, glibenclamide is effective on anion currents but failed to block the KORC. Glibenclamide also did not inhibit significantly AHAS (Fig. 2) and thus failed to induce any herbicidal effect as reported for other SU. Thus, the glibenclamide-induced cell death of *A. thaliana* suspension cells, mimicking the $HrpN_{ea}$-induced cell death, most likely involves the inhibition of anion channels. The protecting effect of bromotetramisole (5 µM) against the lethal effect of $HrpN_{ea}$ reinforces this hypothesis. Bromotet-

ramisole is an uncompetitive inhibitor specific for alkaline phosphatase and tyrosine phosphatase. It inhibits a constitutive, membrane associated, phosphatase activity and stabilizes the phosphorylated form of CFTR, thus increasing the activity of CFTR chloride channel [2]. In our model, the stimulation of anion current by bromotetramisole is compatible with such effects since phosphorylation is required for complete activation of slow anion channel [38]. A putative phosphorylated form of anion channels could avoid the $HrpN_{ea}$-induced decrease in anion current, thus counteracting cell death. In conclusion, our data suggest that the anion channel involved in $HrpN_{ea}$-induced cell death is, or is controlled by, a protein exhibiting analogy with the CFTR and highlight the importance of ion channel regulation during the HR.

4. Methods

4.1. Cell culture and voltage-clamp experiments

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 [13] (main ions after 4 days of culture: 9 mM K^+, 11 mM NO_3^- [36]). The pH of the culture medium was maintained at 5.8. Cells were subcultured weekly by a 10-fold dilution. The experiments were conducted on 4-day-old cultures.

For electrophysiological measurements, the cells were impaled in the culture medium as previously described [10,36]. 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 [11]. 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.

4.2. $HrpN_{ea}$ preparation

The harpin was prepared and partially purified as described previously [10,14]. Briefly, *Escherichia coli* strain K38 (pGp1-2; pMAB64) containing the cloned $hrpN_{ea}$ gene [14] was grown up to an optical density of 0.4 at 600 nm. T7 RNA polymerase was induced according to Tabor et al. [41]. The cells were harvested, resuspended in 12 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 and cell death, a cell free preparation of *E. coli* strain K38 (pGp1-2; pT7-7), containing the pT7-7 vector without the hrpN insert, was prepared in parallel to harpin and used as negative control. Protein contents of both preparations were compared on a 12% SDS-polyacrylamide gel and quantified using the method of Bradford [1]. $HrpN_{ea}$ represent 75% of the total protein preparation.

4.3. Detection of cell death

Cell cultures (25 ml) were incubated for 15 min with 0.05% Evans blue after 24 h of treatment with an effector then washed with 200 ml of deionized water to remove the excess and unbound dye. Dye bound to dead cells was solubilized in 50% methanol with 1% SDS for 60 min at 50 °C and quantified at 595 nm [37]. The data represent an increase in cell death determined with regard to basal cell death control level. The control levels correspond to treatment with negative control for HrpN, and/or the solvent used with other effectors, water for bromotetramisole and methanol for glibenclamide. The final concentration of methanol in all assays was 0.1% (v/v).

4.4. Acetohydroxyacid synthase (AHAS) assays

A. thaliana AHAS was purified as described previously [5]. Assays were performed at 30 °C in a mixture containing 50 mM pyruvate, 1 mM thiamine diphosphate, 10 mM $MgCl_2$ and 10 μM flavin adenine dinucleotide in 100 mM potassium phosphate buffer (pH 7.8). A colorimetric assay [40] was employed. Glibenclamide was dissolved in the organic solvent DMSO and added to the assay to a maximum concentration of 400 μM. The final concentration of DMSO in all assays was 2% (v/v).

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