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Effects of extracellular K^+ on grapevine membrane potential as influenced by the antiviral mycophenolic acid. An electrophysiological study

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Abstract: Mycophenolic acid (MPA) is an effective antiviral drug in plants, and its action in modulating the activity of K_{ATP} channels is already known in animals. In the present work an electrophysiological study was carried out to investigate MPA effects on plant K^+ channels, through the measurement of trans-plasma membrane potential in samples of *Vitis vinifera* cv. Sangiovese treated with extracellular K^+. Tests confirmed that the administration of MPA (in preincubated samples or in those maintained under chemical treatment) can reduce the membrane depolarization induced by K^+. However, MPA-induced alteration in membrane potential was sensitive to the K_{ATP} channel opener diazoxide, as well to treatments with guanosine. This result confirms the effectiveness of MPA in influencing K_{ATP} channel activity as well as inhibiting activity of the inward-rectifier potassium ion channel which could be mediated by guanosine depletion induced by MPA.

1. Introduction

Ion fluxes across cellular membranes are known to play the key role in triggering and mediating defense mechanisms in plants, however little data are currently available on ion signatures generated during plant-virus interactions. Changes in K^+ fluxes after virus inoculation may be mediated by depolarization-activated outward-rectifying K^+ channels (Shabala *et al*., 2010).

In animals, the immunosuppressant drug mycophenolic acid (MPA) [(4E)-6-(4-Hydroxy-6-methoxy-7-methyl-3-oxo-1,3-dihydro-2-benzofuran-5-yl)-4-methylhex-4-enoic acid] depletes cellular guanine nucleoside (GN) by inhibition of inosine monophosphate dehydrogenase, modulating the activity of K_{ATP} channels (Li *et al*., 2000). MPA is also an effective antiviral drug in plants such as grapevine (Panattoni *et al*., 2007; Skiada *et al*., 2009; Luvisi *et al*., 2012 a; Skiada *et al*., 2013; Panattoni *et al*., 2014; Guazzelli *et al*., 2015), but no reports about its effect on K-conducting ion channels in plants are available.

In terms of metabolic dependence of MPA action in plant cells, antiviral drug translocation across membranes was linked to the free energy available in a proton electrochemical potential difference (Luvisi *et al*., 2012 b). Nowadays, compounds that influence K_{ATP} channel activity are currently available for clinical use, and include diazoxide (DO) (7-Chloro-3-methyl-4H-1,2,4-benzothiadiazine 1,1-dioxide) (Babenko *et al*., 1998; 2000) whose effectiveness was also confirmed in plants to investigate the inhibition of mitochondrial K_{ATP} channels (Chiandussi *et al*., 2002).

With regard to the potential effect due to GN depletion by MPA, cyclic derivatives of GN can be involved in plant K-conducting ion channels. In fact, inward-rectifier potassium ion channels have been cloned from *Hordeum vulgare*, *Nicotiana tabacum* and *Arabidopsis thaliana* (Leng *et al*., 1999). These channels open in the presence of cyclic nucleotides such as cyclic guanosine monophosphate (cGMP), and are therefore referred to as cyclic nucleotide gated channels (Leng *et al*., 2002). The cGMP is thought to be present in the cytosol of plant cells, and to be involved in signal transduction pathways which regulate many aspects of cellular metabolism (Assmann, 1995).

The activity of antiviral drugs in grapevine cells was recently investigated using electrophysiological methods (Panattoni *et al*., 2013 a). In the present paper we investigate the effects of MPA on depolarization induced by extracellular K^+ in foliar samples of *Vitis vinifera* cv. Sangiovese. This grapevine cultivar is affected by various plant viruses (Rizzo *et al*., 2012; Rizzo *et al*., 2015) and the ef-

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fects of antiviral drugs where investigated (Panattoni *et al*., 2011; Luvisi *et al*., 2011; Panattoni *et al.*, 2013 b). In addition, the effects of GN- or DO- treatments were evaluated in order to investigate the effect of GN depletion caused by MPA or its effects on K_{ATP} channels.

2. Materials and Methods

Plant material

Virus-free (with regard to viruses included in the European Commission directive 2005/43/EC), eight-year-old *V. vinifera* cv. Sangiovese were used for electrophysiological tests. The sanitary condition of each plant was confirmed by RT-PCR (Nakaune and Nakano, 2006; Faggioli *et al*., 2013). Symptomless plants were used. In June 2013, fully expanded leaves were excised and fresh freehand samples (3-5 mm diameter) were cut with an ethanol-cleaned razor blade for testing (Rinaldelli *et al*., 2012; 2014).

Measurement of membrane potential (Em)

For chemical assays (Table 1), preincubation for 1 h in basal solution (BS) or chemical-BS solutions (MPA-BS, GN-BS, DO-BS at 0.5, 1.0, 2.0 mM) adjusted to pH 5.6 with TRIS (2-Amino-2-hydroxymethyl-propane-1,3-diol) was followed by preparation of leaf segments according to Luvisi *et al*. (2012 b). Step 1 for Em measurements was conducted perfusing aerated BS or chemical-BS through the chamber that fixes the sample at a flow rate of 10.0 x $^{10\text{-}3}$ L min^{-1}. The measuring electrodes used were micropipettes (tip diameter < 1µm) obtained from single-barreled borosilicate capillaries (World Precision Instruments, Sarasota, USA) as described in Rinaldelli *et al.* (2012). Insertion of the microelectrodes took place in the central zone of the mesophyll by way of a micromanipulator (World Precision Instruments, Sarasota, USA). Successful microelectrode impalement was determined by rapid attainment of a steady value without subsequent decay (Ober and Sharp, 2003), followed by a stabilized Em for 5 min. Em after stabilization was recorded to evaluate the effect of chemicals on membrane potential. Membrane signal steadiness was calculated considering the number of successful cell impalements out of those attempted (%). Step 2 began after Em stabilization, using solutions enriched with KCl.

Two procedures were carried out in order to investigate the effects induced by extracellular K^+ following MPA treatments. The first procedure was carried out on samples in which treatment involved only the preincubation step (Li *et al*., 2000). Thus, samples were preincubated in MPA-BS, followed by Em measurement under BS (step 1) and K-BS (step 2). In this test, two concentrations of KCl (5, 10 mM) were evaluated. The effect of chemical treatment was calculated considering Em after BS stabilization and maximum Em achieved after K-BS administration, expressed as Δ Em (Table 1). The second procedure was carried out following a conventional electrophysiological approach. In order to evaluate the interference of chemicals (MPA, GN or DO) on effects induced by K^+, tests were carried out using chemical-BS solution both in preincubation and in step 1, while in step 2 the solution was enriched with KCl (chemical-K-BS) at 10 mM (concentration chosen considering results of the first procedure). The interference of chemicals on membrane depolarization induced by K^+ was calculated considering Em after chemical-BS

Table 1 - Solutions used in membrane potential tests

	Chemical assay		
Solution	Abbreviation	Membrane potential (Em)	
$CaCl_2$ 5.0x10^{-4} M, K_2SO_4 2.5x10^{-3} M, MES 5.0x10^{-3} M	BS	Em_{BS}	
BS with MPA	MPA-BS	Em_{MPA}	
BS with Guanosine	GN-BS	Em_{GN}	
BS with Diazoxide	DO-BS	Em_{DO}	
	Extracellular K^+ assay		
Solution	Abbreviation	Membrane potential (Em)	Variation of membrane potential (%)
BS with KCl	K-BS	Em_K	$Em_K - Em_{BS} = \Delta Em_K$
MPA-BS with KCl	MPA-K-BS	$Em_{MPA\text{-}K}$	$Em_{MPA\text{-}K} - Em_{MPA} = \Delta Em_{MPA\text{-}K}$
GN-BS with KCl	GN-K-BS	$Em_{GN\text{-}K}$	$Em_{GN\text{-}K} - Em_{GN} = \Delta Em_{GN\text{-}K}$
DO-BS with KCl	DO-K-BS	$Em_{DO\text{-}K}$	$Em_{DO\text{-}K} - Em_{DO} = \Delta Em_{DO\text{-}K}$
MPA-BS with Guanosine	MPA-GN-BS	$Em_{MPA\text{-}GN}$	
MPA-BS with Diazoxide	MPA-DO-BS	$Em_{MPA\text{-}DO}$	
MPA-GN-BS with KCl	MPA-GN-K-BS	$Em_{MPA\text{-}GN\text{-}K}$	$Em_{MPA\text{-}GN\text{-}K} - Em_{MPA\text{-}GN} = \Delta Em_{MPA\text{-}GN\text{-}K}$
MPA-DO-BS with KCl	MPA-DO-K-BS	$Em_{MPA\text{-}DO\text{-}K}$	$Em_{MPA\text{-}DO\text{-}K} - Em_{MPA\text{-}DO} = \Delta Em_{MPA\text{-}DO\text{-}K}$

stabilization and maximum Em achieved after chemical-K-BS administration, expressed as Δ Em (Table 1).

Preincubation and electrophysiological tests were carried out at 22±0.5°C under light (30 watt m^{-2}). All tests were conducted on 15 healthy or infected samples. Plots are representative of 15 equivalent experiments. Measurements were performed under Faraday cage to protect tests from external radio frequency interference.

Statistical analysis

The effects of treatments on Δ Em were elaborated using Sigma-Plot software (version 11; Systat Software, San Jose, CA). The software was used to perform one- or two-way analysis of variance (ANOVA) in a random design and pairwise multiple comparisons on significant effects and interactions using the Holm-Sidak method. Data expressed in percent were converted to arcsin values. $P \leq 0.05$ was considered to be significant.

3. Results and Discussion

Em measurement in chemical preincubated samples

Foliar samples were preincubated in chemical-BS solutions and membrane potential was measured with microelectrodes. Preincubation with MPA-BS solution did not change the resting membrane potential with regard to the chosen concentration (Table 2). However, MPA seems to interfere with the signal steadiness at 1.0 and 2.0 mM, causing a reduction of successful impalement by 37.9 and 64.7 %, respectively. Assays carried out with BS enriched by GN or DO showed no effect on membrane potential and did not interfere with the steadiness of the signal.

With regard to the effects induced by extracellular K^+, Em measurement was carried out perfusing aerated BS solution throughout the chamber with the fixed sample (step 1), followed by perfusion of K-BS solution (step 2), and recording the depolarization due to K+ treatment. In MPA-preincubated samples, the antiviral drug can cause a differential response to potassium effects, as shown in Figure 1. The cell response confirms the Nernstian changes in diffusion potential due to increased K^+-concentration or activation of K^+ channels.

As reported in Table 3, KCl caused different depolarization according to each concentration, but the preincuba-

Table 2 - Effect of sample preincubation with chemical administered at different concentrations (0.5, 1.0, 2.0 mM) on membrane potential (Em, mV) or membrane potential signal steadiness on samples of *Vitis vinifera* cv. Sangiovese, expressed as % of successful microelectrode impalement

Treatment	Em (mV)	Signal steadiness (%)
BS	-109.5±6.0 a [z]	53.6
MPA-BS (mM)		
0.5	-110.6±7.2 a	51.7
1.0	-110.0±6.9 a	33.3
2.0	-107.6±5.7 a	18.8
GN-BS (mM)		
0.5	-111.6±8.5 a	50.0
1.0	-112.0±9.5 a	48.4
2.0	-109.0±7.0 a	53.6
DO-BS (mM)		
0.5	-110.9±7.8 a	45.5
1.0	-113.5±8.5 a	48.4
2.0	-108.3±7.1 a	51.7

[z] Values in the same column followed by the same letter do not differ significantly according to Duncan's multiple range test ($P \leq 0.05$).

BS= Basal solution; MPA= Mycophenolic acid; GN= Guanosine; DO= Diazoxide.

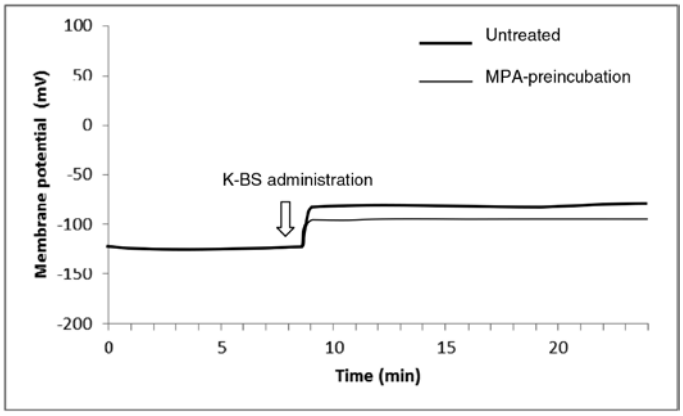

Fig. 1 - Effects induced on samples of *Vitis vinifera* cv. Sangiovese by extracellular K^+ (KCl 10 mM) subsequent to MPA 1.0 mM treatment compared to untreated sample. BS = basal solution; MPA = mycophenolic acid. Plot is representative of 15 equivalent experiments.

Table 3 - Depolarization induced in samples of *Vitis vinifera* cv. Sangiovese by extracellular K+ at 5 or 10 mM following MPA-BS preincubation compared to untreated sample

	Δ Em (%)	
	K-BS (mM)	
	5	10
BS	18.2±3.5a [z] A [y]	30.7±3.7 aB
MPA-BS (mM)		
0.5	17.9±4.1 aA	29.8±3.2 aB
1.0	12.0±3.2 bA	23.3±2.0 bB
2.0	11.1±2.3 bA	16.4±4.1 cB

[z] values in the same column followed by the same letter do not differ significantly according to Duncan's multiple range test ($P \leq 0.05$).

[y] values in the same line followed by the same letter do not differ significantly according to Duncan's multiple range test ($P \leq 0.05$).

The effect was calculated considering Em after BS stabilization and maximum Em achieved after K-BS administration, expressed as Δ Em (%).

BS = basal solution; MPA = mycophenolic acid.

tion with MPA was able to cause a reduction in depolarization. In particular, MPA at 1.0 or 2.0 mM significantly affected KCl at 10 mM, while MPA at 5 mM seemed not to alter the effect induced by extracellular K^+. KCl at 10 mM was used for the subsequent test.

Em measurement in samples maintained under chemical treatment

Tests carried out while maintaining chemical administration throughout steps 1 (perfusion of chemical-BS solution) and 2 (perfusion of chemical-BS solution enriched by KCl) of Em measurement confirmed the effectiveness of MPA in reducing the effect induced by K^+ (Table 4). MPA interference was concentration-dependent and at the lower concentration MPA did not interfere with the effect on membrane potential induced by K^+. Conversely, at higher dosages (2:1 MPA:KCl), MPA causes a $\Delta Em_{MPA\text{-}K}$ of 2.5±0.7%, with a reduction of more than 90% of potassium effects. MPA effectiveness was maintained at equal concentration (1:1 MPA:KCl), showing $\Delta Em_{MPA\text{-}K}$ at 9.5±1.1 %, with a reduction of almost 70 % of potassium effects. No effects on trans-plasma membrane depolarization due to K^+ were registered by GN or DO.

Simultaneous administration of GN with MPA (2:1) caused complete restoration of external K^+ effect on membrane potential, as well as adding DO (1:1) (data not shown).

4. Conclusions

Membrane depolarization caused by MPA (Rinaldelli *et al*., 2012) is a temporary effect. In fact, after 1 h of preincubation in MPA-BS solution, the trans-plasma membrane potential of the sample was not altered by the antiviral drug compared to the untreated control. Similarly, GN or DO did not change resting membrane potential. Interference of signal steadiness caused by MPA at a higher concentration may be linked to cell toxicity induced by the antiviral drug (Panattoni *et al*., 2007; Luvisi *et al*., 2012 a). In fact, samples under stress conditions increase the difficulty of cell membrane measurements (Vuletic *et al*., 1987; Rawyler *et al*., 2002).

With regard to effects induced by extracellular K^+, preincubation tests showed how MPA at 1.0 mM or higher concentrations can interfere up to 10 mM of KCl. The MPA reduction of membrane depolarization caused by extracellular K^+ was confirmed by following tests in which chemical administration was maintained throughout all steps of measurement. The reduction of potassium effects was higher compared to administering MPA only in the preincubation step. This result is probably linked to the variation in MPA intra-/extracellular gradient due to solution changes between preincubation and steps 1-2.

Our results indicate that the antiviral MPA inhibited the effects of extracellular K^+ in plants, through specific channels. In fact, the MPA-induced alteration in membrane potential was sensitive to the K_{ATP} channel opener DO, which hyperpolarized resting membrane potential in treated cells to a level similar to that achieved in control cells; results were also similar to those obtained in animal cells (Li *et al*., 2000). Moreover, GN was able to inhibit the MPA action against extracellular K^+, suggesting that MPA could act also as an inward-rectifier potassium ion channel inhibitor through the depletion of GN.

Inhibition of the activity of K^+ channels caused by MPA may be involved in programmed cell death (PCD) in

Table 4 - Two-way factorial analysis of variance of Δ Em caused by chemical-K-BS (MPA-K-BS, GN-K-BS, DO-K-BS, KCl at 10 mM) on samples of *Vitis vinifera* cv. Sangiovese treaded chemical-BS (MPA-BS, GN-BS, DO-BS). Pairwise multiple comparison analysis with Holm-Sidak test was performed

Source of Variation	DF	SS	P
Treatment (A)	2	0.836	<0.001
Concentration (B)	3	0.228	<0.001
A x B	6	0.696	<0.001
Residual	170	0.207	
Total	181	1.961	
Comparison for factor	DM	t	P
Comparison for A			
DO vs. MPA	0.149	23.461	<0.001
GN vs. MPA	0.139	21.880	<0.001
DO vs. GN	0.010	1.5888	NS
Comparison for B within MPA			
0.0 mM vs. 2.0 mM	0.284	22.276	<0.001
0.5 mM vs. 2.0 mM	0.250	19.586	<0.001
0.0 mM vs. 1.0 mM	0.231	18.160	<0.001
0.5 mM vs. 1.0 mM	0.197	15.470	<0.001
1.0 mM vs. 2.0 mM	0.052	4.116	<0.001
0.0 mM vs. 0.5 mM	0.034	2.690	NS
Comparison for B within GN			
2.0 mM vs. 0.5 mM	0.00543	0.433	NS
2.0 mM vs. 0.0 mM	0.00467	0.366	NS
1.0 mM vs. 0.5 mM	0.00276	0.220	NS
2.0 mM vs. 1.0 mM	0.00267	0.209	NS
1.0 mM vs. 0.0 mM	0.00200	0.157	NS
0.0 mM vs. 0.5 mM	0.00076	0.061	NS
Comparison for B within DO			
2.0 mM vs. 0.5 mM	0.0473	3.771	NS
2.0 mM vs. 0.0 mM	0.0447	3.505	NS
2.0 mM vs. 1.0 mM	0.0406	3.189	NS
1.0 mM vs. 0.5 mM	0.0067	0.531	NS
1.0 mM vs. 0.0 mM	0.0040	0.316	NS
0.0 mM vs. 0.5 mM	0.0026	0.210	NS

BS= Basal solution; MPA = Mycophenolic acid; GN = Guanosine; DO = Diazoxide.
SS= sum of square, DF = degrees of freedom, DM = difference of means, t = t-value.
NS= Non significant at P> 0.05.

response to viral infection. Excessive K^+ efflux and intracellular K^+ depletion are the key early steps in apoptosis (one form of the PCD) in mammalian systems. A critical role for potassium homeostasis in the apoptotic process has also been endorsed for plants (Huh *et al*., 2002; Shabala *et al*., 2007; Shabala, 2009) even if it has not yet been linked to the mechanism of action of MPA in plants.

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