Effects of ion channel inhibitors on cold- and electrically-induced action potentials in *Dionaea muscipula*

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Abstract

Glass microelectrodes were inserted into *Dionaea muscipula* (Venus flytrap) lobes and the action potentials (APs) were recorded in response to a sudden temperature drop or a direct current (DC) application. The effect of potassium channel inhibitor, tetraethylammonium ion, was the lengthening of the depolarization phase of AP. APs were also affected by the anion channel inhibitor, anthracene-9-carboxylic acid, that made them slower and smaller. Neomycin, which disturbs inositol triphosphate-dependent Ca^{2+} release, caused the visible inhibition of AP, too. Ruthenium red, which blocks cyclic ADP-ribose-dependent Ca^{2+} release, totally inhibited DC-triggered APs and induced the decrease in the amplitudes of cold-evoked APs. Lanthanum ions significantly inhibited both cold- and DC-induced membrane potential changes. It was concluded that during excitation *Dionaea muscipula* relied upon the calcium influxes from both the extra- and intracellular compartments.

Additional key words: calcium, cyclic ADP-ribose, inositol triphosphate, phospholipase C, tetraethylammonium ion, Venus flytrap.

Introduction

The action potentials (APs) play an indispensable role in rapid plant movements. During the depolarization phase of AP, an increase in the cytosolic free calcium ($[Ca^{2+}]_{cyt}$) occurs. AP-dependent $[Ca^{2+}]_{cyt}$ increase was demonstrated first in algal cells (Williamson and Ashley 1982, Kikuyama and Tazawa 1983, Beilby 1984) and then in the terrestrial liverwort *Conocephalum conicum* (Trebacz *et al.* 1994). For *Dionaea muscipula* indirect evidence of $[Ca^{2+}]_{cyt}$ increase was provided by Hodick and Sievers (1986). With increasing external Ca^{2+} concentration the amplitude of AP grew while the duration shortened. As a conclusion, the dual function of the calcium increase was postulated: *1*) Ca^{2+} as a main electrical charge carrier responsible for AP depolarization, and *2*) Ca^{2+} as a second messenger in pressure potential regulation (Hodick and Sievers 1986). The role for AP-coupled Ca^{2+} as a second messenger between the membrane depolarization and a physiological response has been proved recently by Fisahn *et al.* (2004). In *Solanum tuberosum*, internal Ca^{2+} release was responsible for activation of plant defence and only AP with a sufficient Ca^{2+} influx relayed information for the specific response (Fisahn *et al.* 2004).

The source of Ca^{2+} ions during AP generation is still a matter of dispute. Thiel *et al.* (1997) showed a significant delay of Ca^{2+} influx during AP, suggesting intracellular Ca^{2+} release. Plieth *et al.* (1998) "visualized" by fura imaging Ca^{2+} release from internal stores during excitation of *Chara*. Supporting these findings, Thiel and Dityatev (1998) postulated a stepwise release of Ca^{2+} from internal stores. Biskup *et al.* (1999) reported that a small voltage-dependent calcium influx started the cascade of AP by activating phospholipase C (PLC) and consequently inositol triphosphate (IP_3)-induced mobilization responsible for calcium induced calcium release (CICR). Wacke and Thiel (2001) provided the basis for a model describing voltage-dependent IP_3 production as a link between electrical stimulation and Ca^{2+} mobilization. The validity of that model was questioned by Tazawa and Kikuyama (2003), who stated that the AP-associated increases in $[Ca^{2+}]_{cyt}$ were mainly due to an increased influx of Ca^{2+} through the plasma membrane. Even though CICR was not ruled out, Tazawa and Kikuyama (2003) postulated that the contribution of

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Abbreviations: A - amplitude; AP - action potential; A-9-C - anthracene-9-carboxylic acid; cADPR - cyclic ADP-ribose; CICR - calcium induced calcium release; IP_3 - inositol triphosphate; PLC - phospholipase C; RP - resting potential; RyR - ryanodine receptor; $t_{1/2}$ - half-time of action potential; TEA^+ - tetraethylammonium ion.
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internal Ca^{2+} release reinforcing initial Ca^{2+} influx from the apoplast during excitation was minor and could not account for any physiological response.

Dionaea muscipula is a carnivorous plant for which APs play a key role in a trap closure. The trap-lobes of this carnivorous plant can be regarded as an assemblage of excitable cells. APs spread over the lobe with a velocity of 10 cm s^{-1} (Sibaoka 1966) and last no longer than 5 s. Although two succeeding APs trigger a trap closure (Hodick and Sievers 1988), the trap can generate over 100 APs in a succession (Trebacz *et al.* 1996). Neither the duration nor the amplitude of APs is altered by the closure that usually takes place within 0.5 s (Hodick and Sievers 1989). In *Dionaea*, APs have been triggered by mechanical stimulation of a receptor hair (Hodick and Sievers 1986, 1988, 1989), by electrical stimulation (Trebacz *et al.* 1996), or by light (Trebacz and Sievers 1998). In this study we demonstrate that a temperature drop can evoke APs, too. The study was to obtain more information about the mechanism of APs in plants. Hodick and Sievers (1986) demonstrated that in *Dionaea* the AP amplitudes depend mainly on Ca^{2+}. The aim of our study was to check what is the source of Ca^{2+} entering cytosol during excitation: apoplast, internal stores or both. In addition to different calcium channel modulators we used anion and potassium channel inhibitors in order to check whether the general scheme of plant excitation consisting of a transient activation of Ca^{2+}, Cl^-, and K^+ channels, respectively (Lunevsky *et al.* 1983, Kikuyama *et al.* 1983, 1984), applies to *Dionaea*.

Materials and methods

Dionaea muscipula Ellis plants grown in a vegetation room were used. A whole lobe cut off the trap was immobilized and immersed into the standard solution containing 1 mM KCl, 1 mM $CaCl_2$, 50 mM sorbitol (pH 7.2). Electrophysiological experiments were performed as previously described (Trebacz *et al.* 1996, Trebacz and Sievers 1998). The transmembrane potential of excitable cells was measured with glass micro-electrodes (*Hilgenberg*, Malsfeld, Germany) filled up with 3 M KCl; Ag/AgCl wires of the microelectrodes were connected to VF-4 buffer amplifier (*World Precision Instruments*, Sarasota, FL, USA). Action potentials were triggered by electrical stimuli up to 4 V. The stimulation circuit consisted of two sharpened silver wires (cathode and anode) impaled in the edge of a lobe and connected to a regulated DC source. A local administration of 0.5 cm^3 of cold solution (1 °C) to the plant surface evoked AP, too. The local drop of temperature was by approx. 10 °C (Krol *et al.* 2003). In order to avoid a gradual decrease in amplitudes (reported after frequent stimulation by Trebacz *et al.* 1996), all stimuli were executed every 5 min, which is much longer than a relative refractory period ranging between 5 and 10 s.

All experiments started after 1-h incubation of the lobe in the standard solution at room temperature (22 - 25 °C). First, a few APs were recorded as a control. Next, the solution was quickly replaced by the one containing additionally: *a*) 2 mM anthracene-9-carboxylic acid (A-9-C); *b*) 10 mM tetraethylammonium ion (TEA^+) - chloride salt; *c*) 0.4 mM verapamil in 0.2 % ethanol; *d*) 5 mM $LaCl_3$; *e*) 2 mM neomycin; *f*) 1 mM ruthenium red; *g*) 5 mM $SrCl_2$; *h*) 2 mM caffeine. A-9-C is an anion channel inhibitor commonly used for the inhibition of the depolarization phase of AP (Trebacz *et al.* 1997, Krol and Trebacz 1999, Krol *et al.* 2003). TEA^+ blocks voltage-dependent potassium channels. Verapamil inhibits L-type Ca^{2+} channels, while La^{3+} ions are known to block a variety of Ca^{2+} channel types. Neomycin suppresses phospholipase C, PLC, and thus stops IP_3-induced calcium release from internal stores (Zhang *et al.* 2002). Ruthenium red is a well-known inhibitor of cyclic ADP-ribose-activated Ca^{2+}-channels (Muir and Sanders 1996) and also disturbs CICR (Willmott *et al.* 1996, Muir *et al.* 1997, Bauer *et al.* 1998). Strontium ions and caffeine are activators of Ca^{2+} channels of a cyclic ADP-ribose-gated type (Allen *et al.* 1995, Bauer *et al.* 1998). An electrical one always accompanied cold stimulation in order to compare the effects of the chemicals used on the records of APs triggered by different stimuli.

The chemicals had no effect on a membrane resting potential (RP), if not mentioned otherwise. The values of parameters are given as means ± SE. In order to establish whether the treatment had a significant effect on AP or not, *t*-test and *ANOVA* were applied. Because *ANOVA* showed that the general difference exists, the additional Tukey post-hoc test was performed to assess the difference between the control and the different treatments in *ANOVA* setting (see Table 1).

Results

Generally, the amplitudes of APs varied among individual traps but did not depend on a kind of stimulus. They equalled for electrical and cold stimulation 67 ± 2 mV (n = 166) and 68 ± 1 mV (n = 166), respectively. However, the duration of APs differed considerably depending on the stimulus and was as follows: 1.3 ± 0.06 s for DC (n = 166) and 2.1 ± 0.07 s for a temperature drop (n = 166).

A-9-C caused a significant decrease in AP amplitudes and a simultaneous increase in AP duration regardless a

Table 1. The influence of ion channel modulators on cold-induced and electrically-triggered action potentials in mesophyll cells of *D. muscipula* traps. Parameters characterizing potential changes: A [%] - the average AP amplitude given as % of the control; $t_{1/2}$ [%] - the average AP duration given as % of the control, the half-time of AP is measured in the half of the amplitude; $_{cold}$ - refers to a low temperature stimulation; $_{DC}$ - refers to an electrical stimulation; n = 10 - 32 (records of APs); * - statistically significant difference.

	A_{cold} [%]	$t_{1/2\ cold}$ [%]	A_{DC} [%]	$t_{1/2\ DC}$ [%]
2 mM A-9-C	67 ± 4*	157 ± 13*	18 ± 5*	226 ± 11*
	P = 0.03	P = 0.04	P = 0.00	P = 0.00
10 mM TEA	149 ± 5*	207 ± 4*	98 ± 2	129 ± 3*
	P = 0.00	P = 0.04	P = 0.99	P = 0.03
5 mM $LaCl_3$	45 ± 5*	159 ± 8*	69 ± 5*	125 ± 9
	P = 0.00	P = 0.04	P = 0.05	P = 1.00
0.4 mM verapamil	101 ± 6	90 ± 11	106 ± 4	103 ± 10
	P = 1.00	P = 1.00	P = 0.99	P = 0.99
2 mM neomycin	82 ± 4*	75 ± 24	74 ± 3*	170 ± 6*
	P = 0.02	P = 0.93	P = 0.02	P = 0.00
1 mM ruthenium red	80 ± 5*	170 ± 20*	0*	0*
	P = 0.01	P = 0.00	P = 0.00	P = 0.00
5 mM $SrCl_2$	135 ± 8*	357 ± 14*	112 ± 2*	123 ± 6
	P = 0.00	P = 0.00	P = 0.04	P = 0.93
5 mM caffeine	106 ± 6	143 ± 10*	103 ± 3	239 ± 10*
	P = 0.99	P = 0.03	P = 0.99	P = 0.00

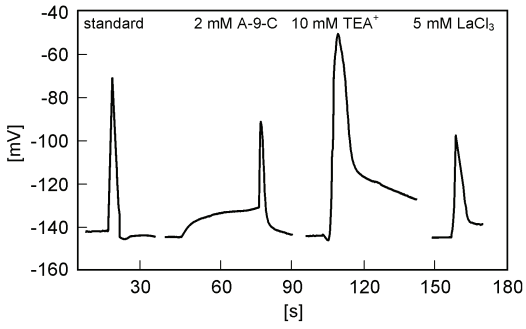

Fig. 1. Exemplary traces of cold-evoked APs in *Dionaea muscipula* lobes immersed for 2 h in: standard solution; standard solution + anion channel inhibitor (2 mM A-9-C); standard solution + potassium channel inhibitor (10 mM TEA^+);; standard solution + calcium channel inhibitor (5 mM $LaCl_3$).

stimulus kind (Table 1). In most of the records, the depolarization phase was clearly shortened and slowed down (Fig. 1).

Addition of 10 mM TEA^+ to the standard medium caused a significant increase in the cold-induced AP amplitudes (Fig. 1). Although it did not have the same effect on the DC-triggered AP amplitudes, in both cases, *i.e.* in the case of electrical as well as cold stimulation, TEA^+ made APs long lasting (Table 1). The repolarization of AP took longer time in the presence of TEA^+ than in the standard solution (Fig. 1).

Verapamil, when added to the standard medium, affected neither AP amplitude nor AP duration (Table 1). La^{3+} ions influenced the amplitude of both cold- and DC-induced APs (Table 1). They also widened the cold-induced APs pronouncedly (Fig. 1). Such an increase in the duration was not so obvious in the case of the DC-evoked APs (Table 1). The inhibitory effect of 5 mM $LaCl_3$ was already visible after one hour treatment.

In order to check a possible calcium-induced calcium release (CICR) we used various substances that affect intracellular calcium fluxes. Addition of 2 mM neomycin to the bath solution resulted in a significant inhibition of the AP amplitudes in all cells examined (Table 1, Fig. 2). However, 2 mM neomycin also caused a significant depolarization of the resting potential (RP). During 3-h-long experiments, RP changed from -125 ± 1 mV to -114 ± 2 mV (n = 6). Apart from the amplitude, neomycin influenced the duration of DC-triggered APs. After electrical stimulation the APs lasted two times longer than the control ones (Table 1). The duration of cold-induced APs in 2 mM neomycin did not differ from the duration of APs in the standard solution.

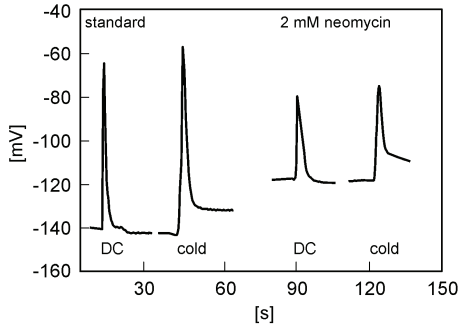

Fig. 2. The comparison of individual cold- and electically-triggered APs and the effects of 2 mM neomycin (PLC-inhibitor) on AP records.

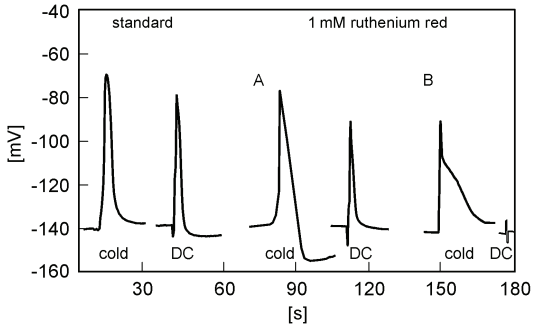

Fig. 3. The effects of 1 mM ruthenium red on DC- and cold-induced APs during the first (*A*) and the third hour (*B*) of experiment.

Ruthenium red (1 mM) caused the gradual inhibition of AP amplitudes and, in the case of DC-triggered APs, produced a total inhibition of AP (Table 1). During the first hour of the application of ruthenium red, the amplitude of DC-induced APs decreased to 71 ± 3 % (n = 20) of the control (Fig. 3*A*). In the same time, the cold-evoked APs were also slightly decreased (91 ± 1 % of the control, n = 10) and substantially widened (116 ± 4 %

of the control, n = 10), (Fig. 3*A*). After 3-h treatment, the inhibition of AP amplitudes was more obvious (Fig. 3*B*). The depolarization phase of both DC- and cold-induced AP was lengthened under the influence of ruthenium red (Fig. 3).

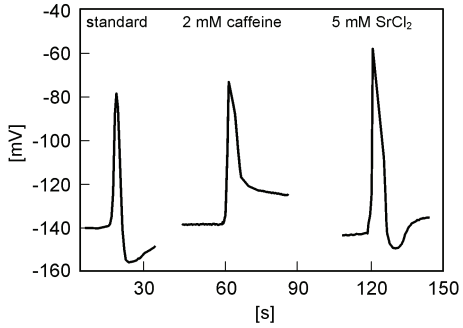

Fig. 4. The exemplified influence of 2 mM caffeine and 5 mM strontium on cold-evoked APs in *Dionaea muscipula* trap cells.

The pronounced increase in the duration of cold-evoked APs was also observed after an addition of 5 mM $SrCl_2$ or 2 mM caffeine (Fig. 4). The duration of DC-induced APs was only affected by caffeine. Thus, caffeine slowed down both, cold- and DC-triggered APs, while strontium widened only the APs evoked by a temperature drop. Caffeine had no effect on the AP amplitudes (Table 1), whilst Sr^{2+} ions influenced the AP amplitudes causing their significant growth (Fig. 4).

Two long-lasting controls (up to 5 h) - in the standard solution and the standard solution supplemented with 0.2 % ethanol - were performed. The former proved that either RP or AP records did not significantly changed throughout the experiment. The latter showed that the ethanol influenced neither the membrane potential nor the AP amplitudes.

Discussion

APs in higher plants provide the fastest mechanism for long-distance signalling (Dziubinska 2003). Its mechanism elaborated for Characean algae still lacks the detailed indication of ionic currents that precede increased $[Ca^{2+}]_{cyt}$. Our results are in agreement with the involvement of calcium fluxes during initial membrane depolarization that is next dominated by chloride efflux and finally reversed by potassium loss. A significant increase in the cold-induced AP amplitudes and even more pronounced growth in the AP duration after the addition of TEA^+ points to the dependence of the depolarization phase on potassium ions. TEA^+ clearly blocks potassium channels and thus does not allow K^+ ions to limit AP depolarization, as postulated by Trebacz *et al.* (1997).

We argue that the depolarization phase of AP in *Dionaea muscipula* comprises calcium and chloride fluxes, not just calcium influx as postulated by Hodick and Sievers (1986). Because of the activation of calcium-gated chloride channels during AP, the amplitude of AP depolarization should mainly depend on a transmembrane gradient for Ca^{2+} ions and only partially on Cl^- ones. The former dependence was shown by Hodick and Sievers (1986). The partial Cl^- dependence can be assumed, since the inhibition of AP amplitudes in the presence of A-9-C was never complete (Fig. 1). We conclude that apart from Ca^{2+}, Cl^- ions as well are the part of depolarizing flow.

Lots of studies implicate Ca^{2+} as a second messenger in abiotic stress responses of plants (Sanders *et al.* 1999, 2002, Knight and Knight 2001, Xiong *et al.* 2002). One of the primary steps in cold perception by higher plants involves Ca^{2+} channel activation, transient rise in $[Ca^{2+}]_{cyt}$, and transient membrane depolarization (Plieth *et al.* 1999, Knight 2002, Krol *et al.* 2004). Knight (2002) reported that after cooling, calcium is transferred into the cytosol in two steps: the initial - very rapid one - involves Ca^{2+} influx from external spaces, whereas the second resembles much slower flux from the vacuole.

Conocephalum conicum generates APs in response to a temperature drop (Favre *et al.* 1999, Krol *et al.* 2003), and so does *Dionaea muscipula*. According to our findings, external and internal Ca^{2+} stores are both engaged in the cold-induced AP of *Dionaea muscipula*. This was demonstrated, on the one hand, in experiments with La^{3+} ions - inhibitors of plasma membrane calcium channels, and on the other hand, after the application of factors modulating Ca^{2+} release from internal stores. The drastic inhibition of AP amplitudes by lanthanum (Fig. 1) strongly argues for calcium influx from the apoplast. These data are in agreement with previous reports (Iijima and Sibaoka 1985, Hodick and Sievers 1986, 1988, 1989). However, a complete inhibition was never observed, either during here-presented experiments or in those with *Conocephalum conicum* (Krol *et al.* 2003). For *Solanum tuberosum*, $LaCl_3$ was not effective in inhibiting the rise in $[Ca^{2+}]_{cyt}$ during an elicitor-induced AP, either (Fisahn *et al.* 2004). Thus, we suppose that La^{3+} disturbs Ca^{2+} influx from apoplast but CICR is not ruled out in the presence of lanthanum.

Inhibitors of internal calcium channel caused a substantial decrease of AP amplitudes (Figs. 2, 3). Moreover, in the case of ruthenium red, a total inhibition of electrically triggered AP was observed (Fig. 2). The total loss of excitability in the case of electrical stimulation under ruthenium treatment suggests that the DC-triggered APs rely mainly on cADPR-related Ca^{2+} sources and only partially on the others (namely apoplastic and IP_3-dependent ones). This is in opposition to the results Biskup *et al.* (1999), who showed that ryanodine-sensitive Ca^{2+} stores seemed to be irrelevant for electrical excitation in *Chara corallina*. However, the work of Fisahn *et al.* (2004) having dealt with ruthenium red showed, that although RyR-stores were engaged, AP did not ceased in the presence of ruthenium red. Pre-

treatment of potato plants with ruthenium red completely blocked the increase of $[Ca^{2+}]_{cyt}$ during membrane depolarization but made that depolarization only slightly smaller (Fisahn *et al.* 2004; Fig. 3). The slight but significant inhibition of the cold-induced AP amplitudes was also recorded by us (Fig. 3).

For neomycin the inhibition was never complete and was additionally accompanied by RP depolarization. However, lower values of AP peak were always recorded in the presence of neomycin than in the standard bath (Fig. 2). Thus, the simultaneous activation of RyR and IP_3-receptor during AP is not ruled out. As reviewed by Xiong and Zhu (2001) these two Ca^{2+} releasing pathways did not need to be simply redundant.

Additional supports that we were dealing with Ca^{2+} fluxes originating from internal stores during excitation, came from experiments with two different activators of Ca^{2+} channels of a cyclic ADP-ribose-gated type: caffeine and $SrCl_2$. The substantial and significant increase in AP amplitudes in the presence of Sr^{2+} ions (Fig. 4) is in favour of internal Ca^{2+} release at the time of AP. Such an effect has already been reported for *Conocephalum conicum* (Krol *et al.* 2003).

The internally-originated Ca^{2+} fluxes are supertuned by an array of different processes such as: ligand-gated channel activation, protein phosphorylation/dephosphorylation, cytoskelatal rearrangements, activities of Ca^{2+}-ATPases and Ca^{2+} transporters, capacities of Ca^{2+} sensing proteins (Sanders *et al.* 1999, 2002, Anil and Rao 2001, Ng and McAinsh 2003). Our results suggest that during the excitation of *Dionaea*, an increase in $[Ca^{2+}]_{cyt}$ may indeed be administrated by such messengers as cADPR, IP_3 and Ca^{2+}. In *Dionaea muscipula*, Trebacz *et al.* (1996) published the effects of Ca^{2+}-ATPase inhibitors on the AP amplitude and duration. A similar phenomenon was also recorded in this study; disruption in calcium "homeostasis during excitation" resulted in lengthening of AP duration (Table 1). Since some of the chemicals we used should affect Ca^{2+} fluxes across internal membranes, our results indicate that there can be a relation between plasma membrane potential changes and the increase in $[Ca^{2+}]_{cyt}$. The correlation is not direct. However, with a microelectrode tip located in a cytosol, one can detect $[Ca^{2+}]_{cyt}$ changes indirectly as long as the permeability of the plasma membrane for Ca^{2+} dominates the membrane conductance (Krol *et al.* 2004).

The abundant temperature sensors – such as the photosynthetic apparatus (Huner *et al.* 1998), the lipid membrane domains (Murata and Los 1997, Ovary *et al.* 2000), the stress-sensitive membrane spanning proteins (Browse and Xin 2001, Zhu 2001) and various calcium channels (Monroy and Dhindsa 1995, Plieth 1999, White *et al.* 2002) – seem to play an equivalent role in a complex response to cold (Chinnusamy *et al.* 2004). The pathways of Ca^{2+} mobilization in response to cold are probably complex as well and that is why none of the inhibitors used was able to block cold-induced AP completely (contrary to a total inhibition of DC-triggered AP by ruthenium red). Our findings, though indirectly, point to the calcium influx from both, the apoplast and intracellular stores during AP. Because different treatments affected cold- and DC-induced APs differently (Table 1), our results are also in an agreement, that Ca^{2+} fluxes may result from activation of various sources/pathways depending on a kind of a stimulus. These Ca^{2+} fluxes should have different biological significance and result in different outputs, as highlighted by Xiong *et al.* (2002).

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