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The combined effect of Cd^{2+} and ACh on action potentials of *Nitellopsis obtusa* cells

Research Article

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Abstract: Interrelations between the action of acetylcholine (ACh) and cadmium ions (Cd^{2+}) on bioelectrogenesis of *Nitellopsis obtusa* cells were investigated. We analyzed repetitively triggered action potentials (AP), their reproducibility, shape and dynamics of membrane potential after AP induction. ACh significantly increased membrane permeability only at high concentrations (1 mM and 5 mM). Repolarisation level of action potential after the first stimulus was much more positive in all cells treated with ACh as compared to the control. Differences of membrane potentials between points just before the first and the second stimuli were 23.4±.0 mV (control); 40.4±5.9 mV (1 mM ACh solution) and 57.7 ± 8.5 mV (5 mM ACh solution). Cd^{2+} at 20 μM concentration was examined as a possible inhibitor of acetylcholinesterase (AChE) *in vivo*. We found that cadmium strengthens depolarizing effect of acetylcholine after the first stimulus. The highest velocity of AP repolarization was reduced after ACh application and Cd^{2+}strengthened this effect. There were no differences in dynamics of membrane potential after repetitively triggered action potentials in ACh or ACh and Cd^{2+} solutions. This shows that cadmium in small concentration acts as inhibitor of acetylcholinesterase.

Keywords: *Action potential • Acetylcholine • Plant acetylcholinesterase • Plant electrical signaling • Characeae*

1. Introduction

Acetylcholine (ACh) is a phylogenetically ancient molecule, functioning as a local mediator as well as a neurotransmitter in almost all life-forms on earth [1]. ACh is also synthesized in plants, where it is likely involved in the transport of water, electrolytes and nutrients [2]. As an intracellular molecule ACh can control basic cell functions such as proliferation and differentiation. Members of cholinergic system – cholineacetyltransferase and acetylcholinesterase (AChE), are widely distributed among plants too [3]. Most reports concerning the plant ACh, its related molecules and the so called "ACh-binding sites" have suggested that an ACh-mediated system might play a role in the plant response to environmental stimuli. However, the ACh-mediated system and its role in plant's signaling are not yet fully understood. It is unclear whether these compounds play a metabolic or a signaling role. If ACh may cause changes in membrane permeability similar to those found in the excitable membranes of animal cells, then ACh can interfere with electrical cellular signaling pathway in plants too.

To investigate the role of ACh as a signaling molecule in plant kingdom we have used Characeaen cell as a model system. The results obtained from experiments using Characean cells have formed a basis for the studies of plant membrane functions [4]. Basic knowledge on

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bioelectric phenomena of plant membranes has been greatly indebted to internodal cells of charophytes.

Bioelectrical signals have been shown to be widespread in the plant kingdom. Electrical signals play an important role in triggering photosynthetic response across long distances within the plant, and may regulate a variety of physiological responses in plants, including elongation, growth, respiration and water uptake [5]. Many investigators, however, doubt the signaling value of electrical activity in plants, and the subject remains an area of active research. The ion mechanism of action potentials (AP) was intensely studied in giant charophyta cells [6]. The AP is based on the activity of voltage-gated channels which respond to (and cause) changes in membrane potential [7]. Action potentials involve a transient influx of Ca^{2+} to the cytoplasm, and effluxes of K^+ and Cl^- outside [8]. Calcium is one of the most important signals within the cell, and implicated in a series of responses. The release of Ca^{2+} from internal stores (in plants, mainly the vacuoles) or its entry through the plasma membrane is mediated by strictly controlled transporters which themselves can be modulated by Ca^{2+}_{cyt} in a feedforward or feedback manner. The regulation of transient Ca^{2+}_{cyt} increase during AP is very precise and occurs according to an all- or- none mechanism [9]. The changes in Ca^{2+} homeostasis are reflected in AP generation.

The application of electrophysiological techniques in combination with pharmacological knowledge, derived from studies on signaling events in animal cells, has proven to be very powerful in analysis of plant cell membrane processes [10]. The bioelectrical response of a charophyta cell is rapid and highly sensitive to chemicals. Changes in transmembrane potential difference of *Characeae* cells could be used as the best test of the effect of various compounds and its combinations on the plasma membrane and the whole cell. If ACh acts as signaling molecule in plants, it may cause changes in membrane permeability during AP. The existence of cholinesterase and inhibition of its activity may be reflected by alterations in membrane potential too.

It was shown in mammals that cadmium is a potent blocking agent of cholinesterase at any dose [11] and significantly inhibits AChE activity *in vitro* [12]. Moreover, cadmium is a heavy metal which is classified as a carcinogen in humans and as a toxicant in plants. Cd^{2+} at high concentrations strongly inhibits photosynthesis, respiration, growth and plant development, causing even plant death [13]. Cd^{2+} toxic effects on membrane permeability can be attributed to the influence of Cd^{2+} on the membrane transport systems, that are involved in plant membrane electrogenesis [14]. Cadmium acts not only as a cholinesterase inhibitor, Ca^{2+} channel blocker, but also inhibits the release of acetylcholine by interfering with calcium metabolism in vertebrates [15]. Although the overall reactions of plants and animals to Cd^{2+} may be different, many Cd^{2+}-related processes are common in both types of cells [13]. Therefore, it is interesting to know if the external application of ACh and Cd^{2+} is reflected in the generation of action potential of Characea cells. The goal of the present study was to investigate the interrelations between the action of ACh and cadmium ions on bioelectrogenesis of *Nitellopsis obtusa* cells.

2. Experimental Procedures

Internodal cells of freshwater charophyte *Nitellopsis obtusa* (Devs.) J. Gr. were used throughout the experiments. All experiments were performed at room temperature (20±1°C) and under daylight conditions (500±10 Lx). The internodal cells were isolated from neighboring cells and branchlets. The internodes were kept at least overnight in buffered artificial pond water (APW), containing 0.1 mM KCl, 1.0 mM NaCl, 0.1 mM $CaCl_2$, 2.5 mM TRIS, adjusted to pH 7.2 by HEPES. Cadmium and acetylcholine treatment was carried out in the basic APW supplemented with 20 μM $CdCl_2$ and 0.1-1-5 mM acetylcholine chloride (Sigma).

Cells were placed in a plexiglass chamber and continuously bathed in a flowing solution of APW or test solution at a rate of about 1 ml·min^{-1}. Conventional microelectrode technique was used to measure transmembrane potential [16]. Reference electrode, after filling with 3M KCl in agar-agar jelly, was immersed into the experimental solution near the cell. The microelectrodes with 1μm tip diameter were made from borosilicate glass capillaries (Kwik-FilTM, World Precision Instruments Inc., USA) and filled with 3 M KCl. A microelectrode was inserted into the cell and electrical properties of the PM were measured post 1 h of insertion. Action potentials were elicited 6 times every five minutes in each solution (Figure 1) by injecting depolarizing current (0.1 s duration 1 μA/ cm^2 square pulse) between two pools using Ag/AgCl wires [17].

Usually, we waited for an hour after exogenous application of acetylcholine to provide sufficient time to penetrate into the cell.

Signals were amplified with a WPI DAM50 preamplifier (input impedance - 10^{12} Ω, input leakage current – 50 pA, gain –20^x). Data were A/D converted (16 bits, ADS7805P, Burr-Brown Corporation) and stored on the computer memory for the later analysis.

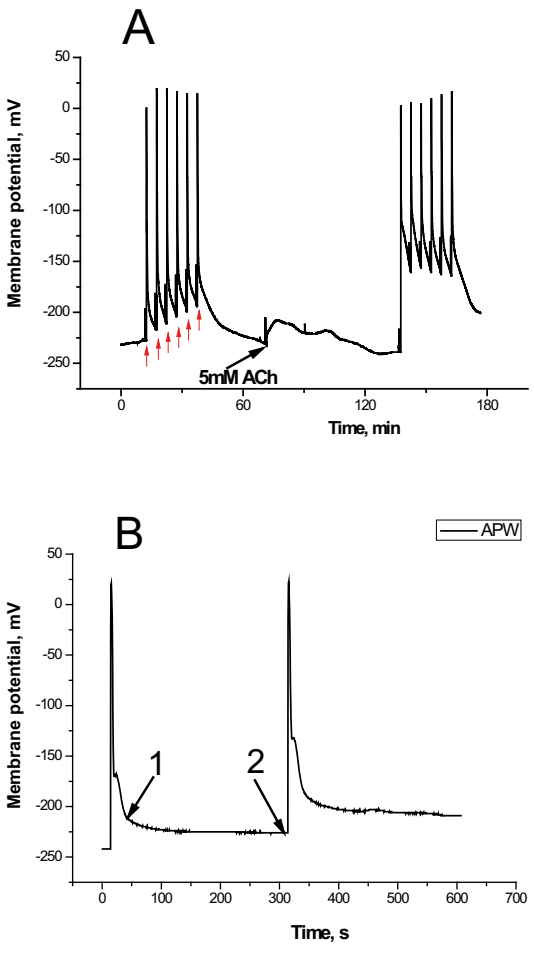

Figure 1. A. Repetitively triggered APs in APW solution (left) and after 5 mM ACh application (right) recorded from *Nitellopsis obtusa* cell. Time between the stimuli (red arrows) is 5 min. Black arrow shows the time of ACh application. Stimulation started after 1 hour. B. First and second APs in APW solution. Arrow "1" shows end of fast repolarization phase, arrow "2" – value of slow repolarization used for calculations.

To evaluate velocity of repolarization we differentiated 50 s periods starting from the peak of AP, took dE/dt and plotted it against voltage. The highest value of dE/dt was taken to evaluate prolongation of repolarization. Data are presented as mean and standard error. Statistical significance of differences was tested using paired T test (n=6) and unpaired T test (n=8). All statements on statistical significance are based on a confidence level of 95%. Calculations and statistical analysis were performed using Microcal ORIGIN 7.5, Statistica 6.0 (StatSoft).

3. Results

3.1 The effect of acetylcholine on single action potential

We investigated the effect of ACh on membrane potential of *Nitellopsis obtusa* cells. We observed that ACh only at high concentrations (1 and 5 mM) caused significant increase in membrane permeability (23%±1.7% and 41%±1.9% respectively). We compared the pattern of single action potentials in APW and 5 mM ACh solutions and found differences in the repolarization phase - ACh modulated repolarization process by increasing its duration (Figure 2).

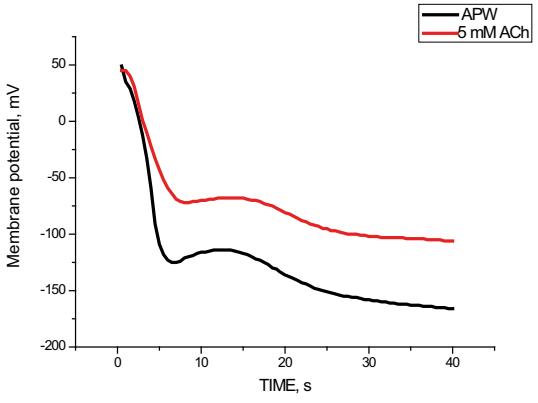

Figure 2. Typical example of 40 s lasting repolarization of AP in APW (black line) and in 5 mM ACh (red line) solution.

To evaluate this effect further, we calculated the highest velocity of fast repolarization and found that it was 63.2 ± 8.2 mV/s in APW and 46.8 ± 7.4 mV/s in 5 mM ACh solution. Slow repolarisation level of action potential 5 min after stimulus application was substantially more positive as compared to the controls in all cells treated with 1 mM and 5 mM concentration of ACh. Differences in membrane potential before and after stimulation were 23.4 ± 5.0 mV in APW, 40.4 ± 5.9 mV in 1mM ACh solution and 57.7 ± 8.5 mV in 5 mM ACh solution. Since ACh modulates slow repolarization we decided to investigate the effect of ACh on membrane potential dynamics after repetitively triggered APs .

3.2 The effect of acetylcholine on the dynamics of membrane potential changes after repetitively triggered APs

Investigating the effect of various chemical compounds on AP of characeaen cells, we observed repetitive firing in *Nitellopsis obtusa* cells when membrane potential

reached certain level of depolarization after application of those compounds. As proposed by V. Sheperd and colleagues [18], this repetitive firing resembles the onset of a critical instability where a time-ordered structure (repetitive firing) emerged after ramping currents to the excitation threshold. Namely, increased background conductance combined with loss of proton pump activity caused gradual depolarization of cell membrane potential to AP threshold. We found that, irrespective of the used solution the average time between spontaneous AP in *Nitellopsis obtusa* cells was 4-5 min. This time is far shorter than it is required for a full repolarization after an electrically induced AP. As in the pump state, resting membrane potential of *Nitellopsis obtusa* cells ranges from -220 mV to -255 mV and is far more negative than AP threshold (about -100 mV). Our decision to evoke repetitive excitation of cells resulting in elevation of membrane potential.We analyzed the repetitively triggered APs, their reproducibility, shape and membrane potential dynamics after AP induction in APW and ACh solution and noticed that ACh caused membrane potential dynamics changes after repetitive excitation in all investigated cells. Changes of membrane potential repolarization after repetitive (every 5 min) excitation of the cell were reproducible in the control solution and could be approximated by the exponent (red lines in Figure 3).

$$y = y_0 + A_1*(1 - \exp(-x/t_1))$$

Where y_0 is membrane potential before stimulation, A_1 describes range of membrane potential dynamics and t_1- time constant (Figure 3). We observed a significant decrease of the time constant from 5.5±1.5 min in APW through 1.5±0.65 min in 1 mM ACh solution down to 0.9±0.35 min in 5mM ACh solution. However, there was no significant difference between the time constant measures in APW and 0.1 mM ACh solution.

As membrane potential after the fifth AP in APW reached plateau, we compared slow repolarization level 5 min after the fifth stimulus (when slow repolarization ended) in all tested solutions. Membrane potentials before the sixth excitation were -192±4.2 mV in APW, -175±5.1 mV in 1 mM, -157±8.3 mV in 5 mM ACh solutions, respectively (P>0.001). In the latter solution, the membrane potential decreased to the same value before the second stimulus applied (Figure 3).

3.3 The effect of cadmium on membrane electrogenesis

We found that cadmium at 100 µM and higher concentrations after 30 min application caused strong depolarization of the plasma membrane (PM) potential in

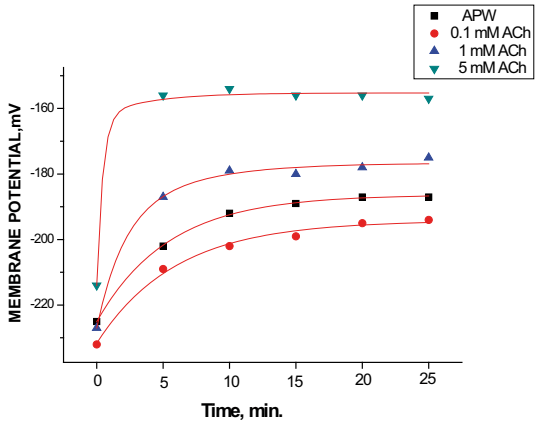

Figure 3. Effect of ACh on dynamics of slow repolarization of average membrane potential after repetitively triggered APs (n=6). Repetitive stimuli were applied every 5 min (see Figure 1). Solid lines - fitted exponential model curves. Slow repolarization was evaluated as the value of membrane potential at the moment of stimulus application, except the value at time 0 min which was resting membrane potential.

all investigated *Nitellopsis obtusa* cells (n=14). In many cases, it led to a spontaneous activity of the cell and a reduction of amplitude of spontaneous AP. Exposure time less than 30 min was not sufficient to evoke spontaneous activity. Electrical stimulation caused reduction of the amplitude of AP in the same manner as in spontaneous AP - amplitude ceased because of decrement of both membrane potential and AP peak (Figure 4). In many cases, cells show spontaneous firing after the end of electrical stimulation. All treated cells died after 2 hours in 100 µM Cd^{2+} solution. Furthermore, electrical stimulation accelerated this process.

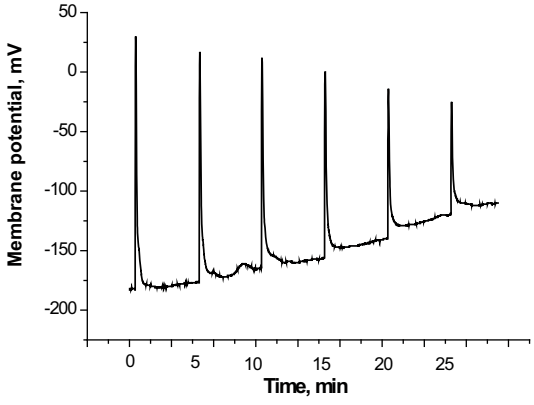

Figure 4. Typical example of membrane potential dynamics after repetitively triggered APs. The cell was treated with with 100 μM Cd^{2+} solution for 20 min before stimulation.

It is considered that strong irreversible depolarization of membrane potential is indicator of Cd^{2+} toxicity [19]. We found that Cd^{2+} at 20 μM did not cause such a depolarization in the majority of tested cells (12 out of 14 cells) with membrane potential more negative than -220 mV. Therefore, we decided to examine Cd^{2+} as a possible inhibitor of AChE *in vivo*.

3.4 The combined effect of cadmium and acetylcholine on membrane potential

We analyzed the effect of cadmium at 20 μM concentration and combined effect of cadmium at 20 μM and acetylcholine at 5 mM concentrations on dynamics of AP. Cells with irreversible depolarization of more than -110 mV and spontaneous firing were excluded from the present analysis. There were no statistically significant differences between the amplitudes of repetitively triggered APs in APW and Cd^{2+} solutions (Figure 4). The amplitude of the first AP after the ACh application did not statistically differ from AP in APW, but there was a distinct decrease in the amplitude of the second AP. Cd^{2+} enhanced this effect on the second AP, while AP amplitude in joint ACh and Cd^{2+} solution statistically differed even after the first stimulus if compared with APW condition. Student t-test proved the differences between AP amplitudes after each stimulus in ACh and ACh+Cd^{2+} solutions were statistically significant (P>0.00001). We found that cadmium strengthens the depolarizing effect of acetylcholine on membrane potential repolarization after the first stimulus (Figure 5).

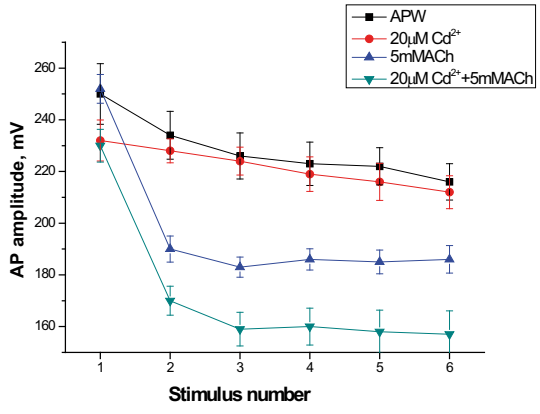

Figure 5. Average amplitude reduction of repetitively triggered APs after in 20 μM Cd^{2+}, 5 mM ACh or 5 mM ACh applied together with 20 μM Cd^{2+} solutions (n=8). Time between stimuli is 5 min. Amplitude was evaluated as difference of membrane potential value before the moment of stimulus application and AP peak.

It is generally accepted that certain ion processes start at a particular value of membrane voltage [20]. Therefore, it was interesting to investigate if the highest repolarization rate in our investigated solution corresponds to the same voltage. To evaluate velocity of repolarization, we differentiated 50 s periods starting from the peak of AP, took dE/dt and plotted it against voltage (Figure 6). We found that the highest rate of repolarization declined after ACh application and Cd^{2+} strengthened this effect. Maximum velocity of potential changes under effect of Cd^{2+} alone was shifted to the more negative potential value as compared with the effect of ACh (Figure 6). The highest value of repolarization rate was 39.6±3.6 mV/s in APW, 33.6±1.8 mV/s in Cd^{2+}, 28.8±3.2 mV/s in ACh 14.9±2.2 mV/s in ACh+Cd^{2+} solutions.

Significant depolarization after application

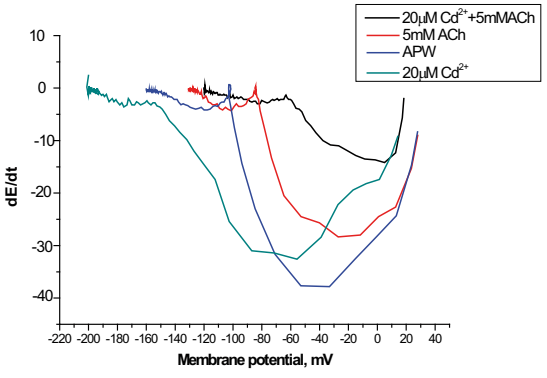

Figure 6. Average phase plane of repolarization dynamics of the second AP after application of 20 μM Cd^{2+}, 5 mM ACh or 5 mM ACh applied together with 20 μM Cd^{2+} (n=8). 50 s period starting from the peak of AP was differentiated to evaluate velocity of repolarization. The dE/dt is plotted against the membrane voltage.

of Ach was reached just after the second stimulus and remained approximately the same through the following stimulations. Therefore, we compared the highest value of fast repolarization rate between the second and last stimuli and found no significant difference (P>0.05) between ACh and ACh plus Cd^{2+} solutions (Figure 7).

4. Discussion

One of the aims of our experiment was to investigate effect of ACh on parameters of action potential in *Nitellopsis obtusa* cells. Our main finding is that, ACh prolongs the repolarization phase of the AP and Cd^{2+} at 20 μM has no effect on the AP by itself, but increases the effect of ACh. Existing experimental data shows that the primary mechanism of action of ACh in plant cells

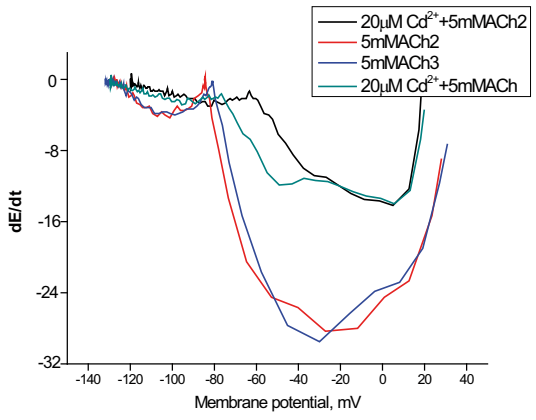

Figure 7. Average phase plane of repolarization of the second (5 mM ACh 2 and 5 mM ACh+20 μM Cd^{2+} 2) and the third (5 mM ACh 3 and 5 mM ACh+20 μM Cd^{2+} 3) AP dynamics after application of ACh or ACh applied together with Cd^{2+}. 50 s period starting from the peak of AP was differentiated to evaluate velocity of repolarization. dE/dt was plotted against the voltage. n=8.

could be *via* the regulation of membrane permeability to protons [21], potassium ions [22], chloride ions [23] and Ca^{2+} [24].

On the other hand, there is experimental evidence that the same transport systems are involved in generation of action potential in plant cells [6,8,25-27]. Our analysis of AP after application of ACh points to the effect of ACh on ion channels which are involved in repolarization of AP. We show that the addition of ACh prolongs the repolarization phase of the action potential in *Nitellopsis obtusa* cells just as Gong and Bisson have demonstrated in *Chara coralina* [23]. They attributed this prolongation to the increased probability of the tonoplast chloride channels opening which would tend to maintain cell in a more depolarized state. The plasmalemma chloride channels which are activated in AP generation may show this effect too. To activate tonoplast channel of *Characeae* ACh must cross the plasma membrane and enter the cytoplasm. The effect of ACh was reversible in our experiments. This supposes involvement of a second messenger system, possibly calcium, activating tonoplast chloride channels or rapid ACh hydrolysis. Effect of high exogenous ACh can be accounted for by its poor penetration into the plants' tissues and/or its rapid hydrolysis. We found maximum effect on AP repolarization at 5 mM ACh concentration for *Nitellopsis obtusa* cells. This observation fits well to the data presented by Gong and Bison [22], where they found maximum effect of ACh on *Chara coralina* cells membrane potential at concentrations ranging from 1 to10 mM.

During the ACh hydrolysis enzymatic cleavage of ACh molecule is performed *via* activity of acetylcholinesterase. It is agreed that AChE is widely distributed in the plant kingdom [28,29]. It can be presumed that inhibition of AChE activity leads to the penetration of exogenous ACh inside the cell, where it can initiate the same processes that are controlled by the endogenous ACh. Indirect possibility of AChE activity in *Characeae* was shown by Dettbarn [30] who found that *Nitella* is capable of hydrolyzing acetylcholine up to 6.5 μmoles/g/h. Based on our data, we suppose AChE activity in *Nitellopsis obtusa* cells as well. The high dose of ACh released inside the cell would have been expected to overcome a possible cholinesterase activity in the cell wall. The presence of acetylcholinesterase in a plant cell capable of propagating electric currents suggests the possibility of a fundamental similarity between the mechanism of bioelectrogenesis in plant and animal cells. The enzymatic activity of AChE has been shown to be altered by environmental contaminants such as metals. It was shown that Cd^{2+} significantly inhibited AChE activity *in vitro* [11].

We performed an *in vivo* experiment aimed to investigate effect of Cd^{2+}on membrane potentials in *Nitellopsis obtusa* cells. Addition of 0.1 or 1 mM Cd^{2+} to the experimental solution caused strong depolarization. Interaction between calcium signaling and cadmium in plant cells has been recently demonstrated in Arabidopsis suspension cells [31]. Decrement of AP peak found in our experiments after Cd^{2+} treatment could be explained by an impact of Cd^{2+} on the intracellular calcium level. Cd^{2+} toxic effects on membrane permeability can be attributed to the influence on the transport systems, involved in plant membrane electrogenesis [14,32,33]. However, it is thought that there may be mechanisms in the plant systems that can resist the toxic effect of Cd^{2+} up to certain concentrations [34]. We found such threshold Cd^{2+}concentration for *Nitellopsis obtusa* cell to be 20 μmol when membrane potential is more negative than -220 mV. In our short lasting experiments (few hours) we assume that Cd^{2+} at 20 μmol/l concentration acts more as acetylcholynesterase inhibitor rather than affecting membrane transport systems at membrane potentials more negative than -220 mV. Membrane potential dynamics after repetitively triggered action potentials shows the same pattern after application of ACh or ACh applied together with Cd^{2+}. In this case the target of action seems to be the same process, and supposedly Cd^{2+} amplifies the action of ACh. We demonstrated that Cd^{2+} strengthens the depolarizing effect of acetylcholine on membrane potential after the first stimulus. It is proposed that, in plants, both frequency and amplitude modulation may be used for encoding of environmental information [35]. It is possible that keeping the cell in

more depolarized state after the first AP in ACh solution has a physiological implication. Probably the second AP causes accumulation of sufficient $Ca^{2+}$for physiological implications. For example, depolarization from more positive membrane potential during the second AP generation consumes less energetic resources. It could be possible that two APs, separated by some critical time interval, have physiological meaning not only in *Dionaea*, [36] but also in other plant species. Assuming a second AP has notional physiological significance, we can propose participation of ACh in electrical signal transmission. Ion fluxes, amplitude, duration and dynamics of repetitively evoked electrical signals can play key roles in generation of physiological responses in plants.

Further research needs to be done to ascertain the effect of specific acetylcholinesterase inhibitors on membrane potential genesis and dynamics in the *Nitellopsis obtusa* cells. The hypothesized effect of Cd^{2+} on the acetylcholinesterase activity could be strengthened by a direct enzymatic assay.

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