VILNIUS UNIVERSITY

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ACETYLCHOLINE EFFECT ON PLASMA MEMBRANE TRANSPORT SYSTEMS OF CHARACEAN CELLS

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VILNIAUS UNIVERSITETAS

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ABBREVIATIONS

ACh – acetylcholine

AChE - acetylcholinesterase

AP - Action potential

APW - control solution (Artificial Pond Water)

ATCh – acetylcholinetransferase

DCCD - Dicyclohexylcarbodiimide

DIM – dimethoate

E_k - potassium equilibrium potential

E_m - membrane potential

H^+-ATPase - proton pump

HEPES - buffer

IP_3 - inositol 1,4,5-triphosphate

K_{in} - inward potassium channels

K_{out} - outward potassium channels

mAChR - muscarinic acetylcholine receptors

MP - membrane potential

Na, K, Cl, Ca, Cd - chemical elements

Na^+, K^+, Cl^-, Ca^{2+} Cd^{2+-} - ions

nAChR - nicotinic acetylcholine receptors

NB - neostigmine bromide

RP - rest potential

SE - standard error

Tris - buffer

1. INTRODUCTION

All biological systems are influenced by their environment. To survive living organisms must perceive environmental changes and synchronize biological function with their responses to the stimuli. At the level of stimulus-response coupling, plants and animal cells undergo many similar processes. Plants as well as animals receive numerous complex environmental information and for its coding use similar signalling systems. While some signal transduction pathways have different roles in plants, much of them are common to all organisms. Not only chemical compounds – nucleic acids, oligonucleotides, proteins, peptides, hormones, some amino acids are involved in plants signal transmission, but also physical - mechanical (especially hydraulic), and electrical signals play role too. Changes in plasma membrane potential or modulation of ion flux are amongst the earliest cellular events in response to environmental stimulation. Recent progress in electrophysiology and molecular biology and genetics has revealed the crucial role of plasma membrane transporters in perception and signalling in response to virtually every known environmental factor (Zimmermann & Sentenac, 1999). The finding that most plant ion channels have homologs in animals has increased interest in plants as model systems. Analysis of differences in plant ion channel gating, kinetics, selectivity, and regulation is contributing to understanding the structure-function relation of ion channels in general. The application of electrophysiological techniques in combination with pharmacological knowledge, derived from studies on signalling events in animal cells, has proven to be very powerful in analysis of plant cell membrane processes. Plasma membrane allows to transmit electrical signals at the cell, tissue and organ level for short and long distances. One of the ways to respond to various stimuli in intracellular and extracellular environment is by activation and propagation of fast electrical signals, i. e. action potentials (Davies, 2004). Characeaen cells are considered unique plant cells, since they generate a large action potential (Beilby, 2007). The giant algae *Nitellopsis obtusa* generates action potentials (AP) in response to mechanical stimulation, injury, or direct electrical stimulation. The bioelectrical response of a Charophyte cell is rapid and highly sensitive to chemicals in environment. Cells of Charophytes are well-characterised experimental systems to study a wide range of membrane transport phenomena. The usefulness of Characeaens in experiments is based

on the ability of internodal cells to survive after isolation from the plant, on the large size and regular shape of these cells. A cell separated from talloma can be considered as a single organism that maintains essential physiological characteristics for a long time (Shimmen et al., 1994). Exploration of Characeaen as a model system helps to understand more complex laws of functionality, adaptation, and information processing in higher plants and animals (Johnson et al., 2002).

The measurements of the bioelectrical response of the cell membrane are one of the most suitable and rapid methods for the investigation of the functionality of membranes and membrane transport systems. Bioelectrical signals have been shown to be widespread in the plant kingdom. Basic knowledge on bioelectric phenomena of plant membranes has been greatly indebted to internodal cells of Charophytes. Although electrical signals in plants have been known for over 100 years, scientists are still looking for a function (Fromm & Lautner, 2005). Many investigators, however, doubt the signalling role of electrical activity in plants, and the subject remains an area of active research. It is established that action potentials actually evoke important responses in plants. Electrical signals play an important role in triggering photosynthetic response across long distances within the plant, and may regulate a variety of physiological responses, including elongation, growth, and respiration and water uptake, regulate the induction of proteinase inhibitor activity and jasmonic acid synthesis (Dziubinska et al., 2003; Fisahn et al., 2004; Grams, et al., 2009).

Combining studies on plant excitability with studies of animal excitability, comparison of plant and animal AP generation and propagation mechanisms may help us better understand the evolution of nervous system (Hille, 2001). From the evolution point of view it is interesting to find in plants many neurotransmitters, neuroregulators and neurotoxins required for the activity of other organisms (Roshchina, 2001). Acetylcholine (ACh) is a phylogenetically ancient molecule, functioning as a local mediator as well as a neurotransmitter in almost all life-forms on earth (Wessler et al., 2003). Biological role of acetylcholine is focused mainly on its neurotransmitter function although the presence of ACh, cholineacetyltransferase and acetylcholinesterase molecules in all forms of life support the idea, that Ach, as local cell molecule, could modulate important physiological functions at the very beginning of life (Horiuchi et al., 2003). Plant cholinergic system has been investigated for more than 30 years (Tretyn &

Kendrick, 1991). 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. ACh is likely involved in the transport of water, electrolytes and nutrients and mediates changes in ion flux across cell membranes (Yamamoto et al., 2008). As an intracellular molecule ACh can control basic cell functions such as proliferation and differentiation (Wessler et al., 2001). Members of cholinergic system cholineacetyltransferase and acetylcholinesterase (AChE) are widely distributed among plants too (Sagane et al., 2005). It also seems probable that acetylcholine acts as a local hormone regulating phytochrome-mediated phenomena and is involved in the regulation of photosynthesis (Wišniewska & Tretyn, 2003). Increase of ACh has been estimated as response to gravity and heat action (Momonoki, 1997)**.** Despite a long history of study, the ACh-mediated system and its role in plant's signalling are not yet fully understood. To date there are no evidence about ACh accumulation mode and balance between ACh synthesis, diffusion and hydrolysis in plants. It is unclear whether these compounds play a metabolic or a signalling role (Kawashima et al, 2007). 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 signalling pathway in plants too.

In the most of plant cholinergic system investigations prevailed detection and structural analysis of distinct components (Horiuchi et al., 2003). Nevertheless, molecular studies alone do not provide information regarding physiological characteristics of ion transport systems in intact cells. Consequently research of plant cholinergic system function and coordination *in vivo* are needed. For such investigations electrophysiological techniques could be useful to determine the effect of ACh on the activity of plant membrane ion transport systems and reveal participation of Ach in signal transduction. To investigate the role of ACh as a signalling molecule in plant kingdom we have used Characeaen cell as a model system.

1.1. The aim and tasks of the study

The aim of the present study was to investigate effect of acetylcholine on the functionality of ion transport systems and dynamics of action potential generation in Characeaen plasma membrane.

The tasks to accomplish the aim of the study were as follows:

1. To investigate the effect of acetylcholine on:
   - basic electrical characteristics in Characeaen *Nitella flexilis* and *Nitellopsis obtusa* cells;
   - single and repetitively triggered action potentials in Characeaen *Nitellopsis obtusa* cells;
   - K^+ channels at the resting state in Characeaen *Nitella flexilis;*
   - Cl^- and Ca^{2+} channels after excitation in Characeaen *Nitellopsis obtusa;*
   - activation of H^+ATPase of Characeaen *Nitella flexilis.*

2. To investigate the effect of acetilcholinesterase inhibitors on changes of electrophysiological characteristics induced by ACh in Characeaen cells.

3. To investigate the influence of ACh agonist nicotine on electrical responses of *Nitellopsis obtusa* cells.

1.2. The novelty and significance of the study

1. It was accomplished for the first time analysis of the acetylcholine effect on the all main membrane transport systems involved in electrogenesis of membrane potential in Characeaen cell, as a model plant system.

2. It was accomplished for the first time the investigation of the effect of acetilcholinesterase inhibitors and nicotine on characteristics of plant action potentials.

3. For the first time repetitively triggered action potentials have been applied for the assessment of the impact of various chemical compounds on the functionality of membrane ion transport systems in *Nitellopsis obtusa*.

Practical application of the study results

It has been shown that analysis of Characeaen membrane potential dynamics triggered by repetitive stimulation could be used in ecotoxicological monitoring.

1.3. Statements to be defended

1. 5 mM ACh activates the main Characeaen membrane transport systems involved in electrogenesis of membrane potential.
2. Acetylcholine and nicotine exert depolarizing effect on membrane potentials in *Nitella flexilis* and *Nitellopsis obtusa* cells.
3. Acetylcholine increases the excitability of *Nitellopsis obtusa* cells.
4. Activity of acetilcholinesterase in Characeaen cells could be revealed by electrophysiological methods *in vivo*.

2. MATERIALS AND METHODS

Internodal cells of freshwater charophyte *Nitellopsis obtusa* (Devs.) J. Gr. and *Nitella flexilis (L.) C. Agardh* were used throughout the experiments. Cells of *Nitellopsis obtusa* were collected in November from Siesartis lake (Lithuania) and have been kept in glass aquarium filled with room temperature ordinary water. *Nitella flexilis* cells were grown in laboratory conditions in bathing medium of 0,1 mM KH_2PO_4, 0,4 mM $CaCl_2$, 1 mM $NaHCO_3$, 0,1 mM $Mg(NO_3)_2$ at 20±1°C temperature, pH 7,2. The cells were subjected to daily cycle of 12 h light and 12 h of darkness. Separate *Nitella flexilis* cells of 4-7 cm length and 0,2-0,4 mm in diameter and *Nitellopsis obtusa* cells of 10-15 cm length and 0,6-0,8 mm in diameter were used throughout research.

The experiments were performed at room temperature (20±1^{o}C) and under daylight conditions (500±10 Lx). To inhibit the light-stimulated electrogenic H^+-ATPase cells were kept in darkness for two or three days. K^+ channels were investigated in the darkness. The internodal cells were isolated from neighbouring cells and branchlets. The internodes were kept at least overnight in buffered artificial pond water (APW), containing 0,1 mM KCl, 1 mM NaCl, 0,1 mM $CaCl_2$, 2,5 mM TRIS, adjusted to pH 7,2 by HEPES or HCl. Acetylcholine, cadmium, neostigmine bromide and nicotine treatment was carried out in the basic APW supplemented with test solutions (Sigma). The electrogenic proton pump of the plasma membrane was inhibited by adding 50 μM dicylcohexylcarbodiimide (Sigma), diluted in 2 ml of ethanol and supplemented with APW till required concentration. 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. Conventional microelectrode technique was used to measure transmembrane potential (Purves, 1981). Reference electrode, after filling with 3 M 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 1 h after insertion. Action potentials were elicited 6 times every five minutes in each solution (**Figure 3.2)** by injecting depolarizing current (0,1 s duration 1 $\mu A/cm^2$ square pulse) between two pools using Ag/AgCl wires. 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 –20x). Data were A/D converted (16 bits, ADS7805P, Burr-Brown Corporation) and stored on the computer memory for the later analysis.

Voltage clamp method was used for the investigation of the activity of separate ion transport systems. Voltage – current characteristics of *Nitella flexilis* plazmalemma were obtained for K^+ ion channels analysis. It is established that K_{in} and K_{out} channels opened at different membrane potential values: K_{out} at -40 mV to -20 mV, K_{in} at -150 mV to -180 mV (Sokolik & Yurin, 1986). Therefore membrane potential was clamped at -40 mV and -160 mV respectively in our experiments. Short (30 ms) rectangular hiperpolarization or depolarization current pulses were injected every 20 mV step. Current curves were displayed on oscilloscope C8-17. Steady voltage – current curves were plotted from the steady state ionic current values against applied voltages.

For the investigation of H^+-ATPase activity in *Nitella flexilis* membrane potential was clamped at -150 mV in the dark (when proton pump is inhibited) and in the light (when light activated proton pump activity was maximal). The difference between voltage-current curves in the dark and in the light conditions was considered as voltage-current characteristic of the H^+-ATPase.

Nitellopsis obtusa cells have been used for the investigation of Cl^- and Ca^{2+} ion channels activity after excitation. Membrane potential was clamped and held in stable state at -180 mV. Rectangular depolarizing current pulses were injected for 25 s in the range from -160 mV to +60 mV every 20 mV. This time is sufficient for activation and

inactivation of Cl^- channels. Data recording was performed using computer software Clampex version 10.2. Data were analyzed using software Clampfit 10.2 .

Voltage value of depolarization step at which the first electrical response was triggered was taken as an excitation threshold. Families of current curves after excitation were obtained by the sequence of single pulses of 20 mV applied every 5 min. The interval between stimulus onset and evoked current response was taken as latency of activation of Cl^- current. Duration of Cl^- current inactivation was calculated as time during which current changed its value from maximal to zero. Reversal potential of Cl^- current was estimated from voltage current curves.

Two distinct components different in time and voltage dependence could be separated in the families of transient current curves registered from the *Nitellopsis obtusa* plasmalemma under stepped depolarizing changes in the holding potential from -20 mV to 40 mV. The quick smaller component arising in 100 ms from the onset of stimulation was taken as Ca^{2+} current, whereas larger and slower component was taken as Cl^- current. Calcium current was measured at 20 mV where it better separates from Cl^- current.

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 and unpaired T test. 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

37 *Nitella flexilis* and 159 *Nitellopsis obtusa* cells were examined in order to investigate the effect of acetylcholine on the activity of ion transport systems and dynamics of action potential generation in characeaen plasma membrane. The measurements were carried out using a conventional microelectrode technique and voltage clamp method. The investigations of the effect of ACh, nicotine and AChE inhibitors have been accomplished.

3.1. The effect of acetylcholine on membrane permeability

The effect of ACh at 0,001 - 5 mM concentrations on membrane permeability of Characeaen cells was evaluated by using conventional intracellular microelectrode technique to measure membrane resistance. It has been ascertained ACh had no toxic effect to all *Nitellopsis obtusa* cells investigated. All cells survived in solutions of 0,1 mM, 1 mM and 5 mM ACh for 7 days. The decrease of cell turgor and alteration in cytoplasmic streaming has not been observed. We estimated that only at high concentrations (1 and 5 mM) ACh caused significant increase in membrane permeability ($p<0,002$, $n=10$) of *Nitella flexilis* and *Nitellopsis obtusa* cells (**Table 3.1**).

Table 3.1. Membrane resistance R, ($k\Omega/cm^2$) of *Nitella flexilis* and *Nitellopsis obtusa* cells after 1 mM and 5 mM ACh application.

Cell / Solution	Membrane resistance R, $k\Omega/cm^2$	
	Nitella flexilis	*Nitellopsis obtusa*
APW	22,3 ± 2,2	23,0 ± 3,9
1 mM	17,6 ± 2,0	17,8 ± 3,0
5 mM	11,7 ± 2,3	13,5 ± 2,5

The effect of ACh was reversible - after removing test solutions resistance reverted from the setting membrane to APW values. Application of 1 and 5 mM ACh decreased membrane resistance in *Nitella flexilis* cells by 20,8% and 47,3% and *Nitellopsis obtusa* cells 22,5% and 41,3% respectively. Increment in membrane permeability caused by solutions where K^+ concentration was increased by 10 times (KAPW solution) was very similar (**Figure 3.1)**.

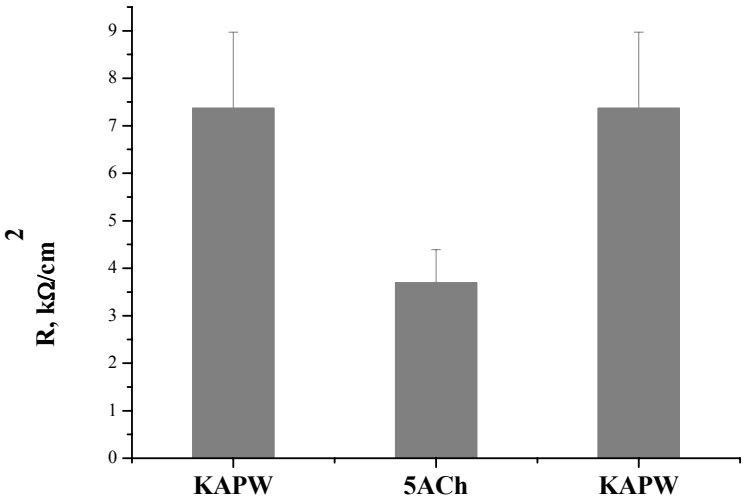

Figure 3.1. Membrane resistance R, ($k\Omega/cm^2$) of *Nitella flexilis* cells after 5mM ACh application in KAPW solution (K^+ concentration was increased by 10 times if compared with APW solution).

Membrane permeability of *Nitella flexilis* cells increased by 49,9% after application of 5 mM ACh in KAPW solution.

3.2. The effect of acetylcholine on membrane rest potential

We found 1-5 mM acetylcholine has depolarizing effect on the resting potential of Nitellopsis obtusa cells. Depolarization started just after application of ACh and steady state was reached in 12 minutes. Gradual hyperpolarization process started and membrane potential reached APW value in approximately 25 min. Dynamics of membrane potential changes is represented in **Figure 3.2.** Resting potential depolarized by 12,2 ± 2,1 mV from -225,3 ± 5,6 mV in 1 mM ACh solution, and by 30,6 ± 3,9 mV from -223,8 ± 4,1 mV in 5mM ACh solution ($p<0,0001$, $n=8$).

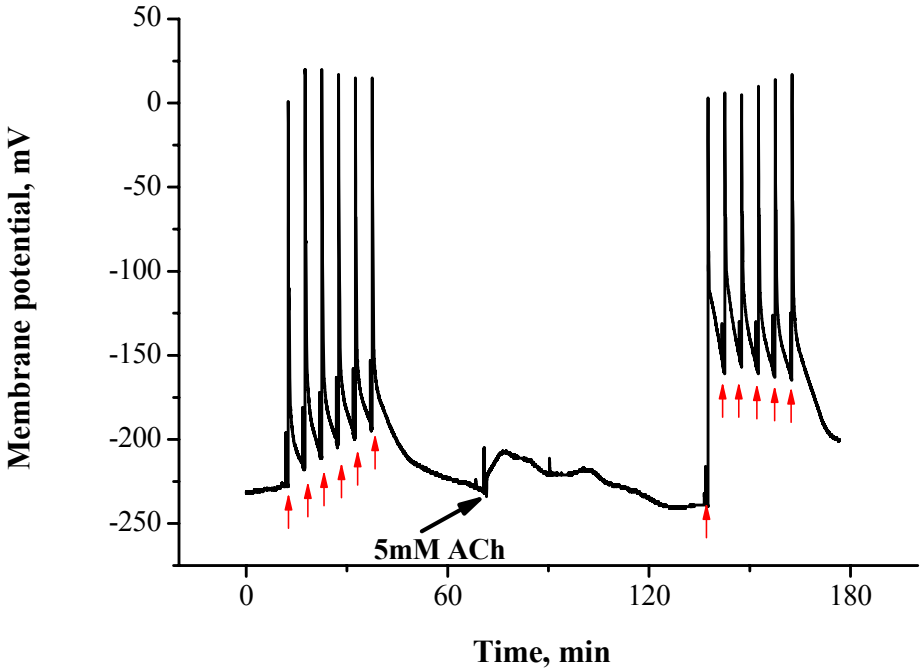

Figure 3.2. Typical example of membrane potential dynamics after repetitively triggered APs in APW solution (left) and after 5 mM ACh application (right) recorded from *Nitellopsis obtusa* cell. Time between the stimuli (vertical arrows) is 5 min. Bold oblique arrow shows time of ACh application (30 min after the last AP). Stimulation in ACh solution started 1 hour after solution application.

3.3 The effect of acetylcholine on single action potential

We compared pattern of single action potentials in APW and 5 mM ACh solutions and found differences in the repolarization phase - ACh modulated repolarization by increasing its duration (**Figure 3.3**).

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 (decreased by 26%). 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 ($p<0,00001$, $n=10$). Differences between membrane potentials before and after stimulation increased by 76% in 1 mM ACh and by 151% in 5 mM solutions (**Figure 3.4)**

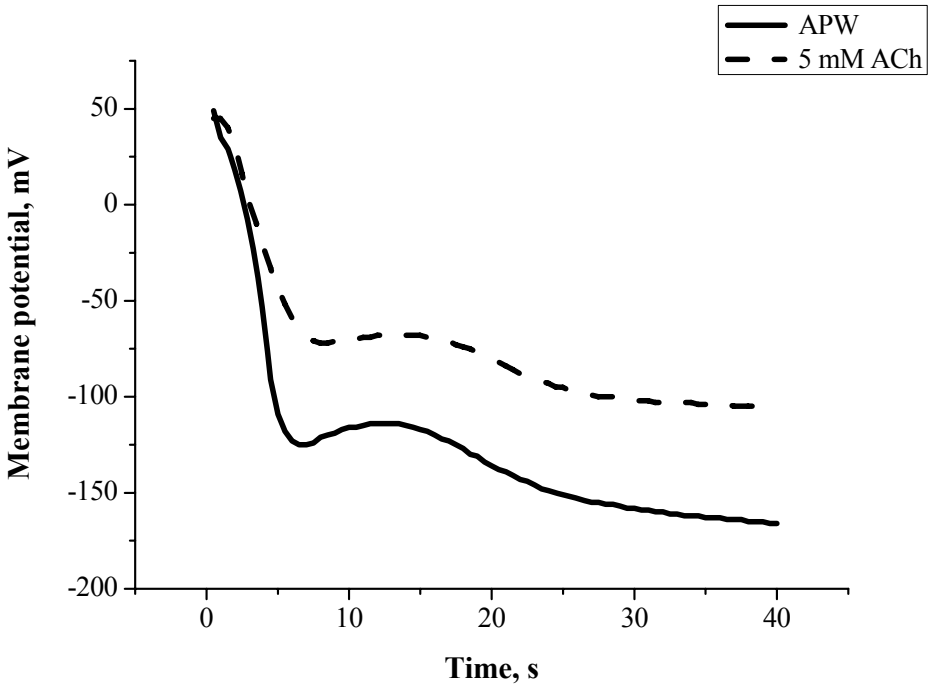

Figure 3.3. Typical example of 40 s lasting repolarization of AP in APW (black line) and in 5 mM ACh (dotted line) solution.

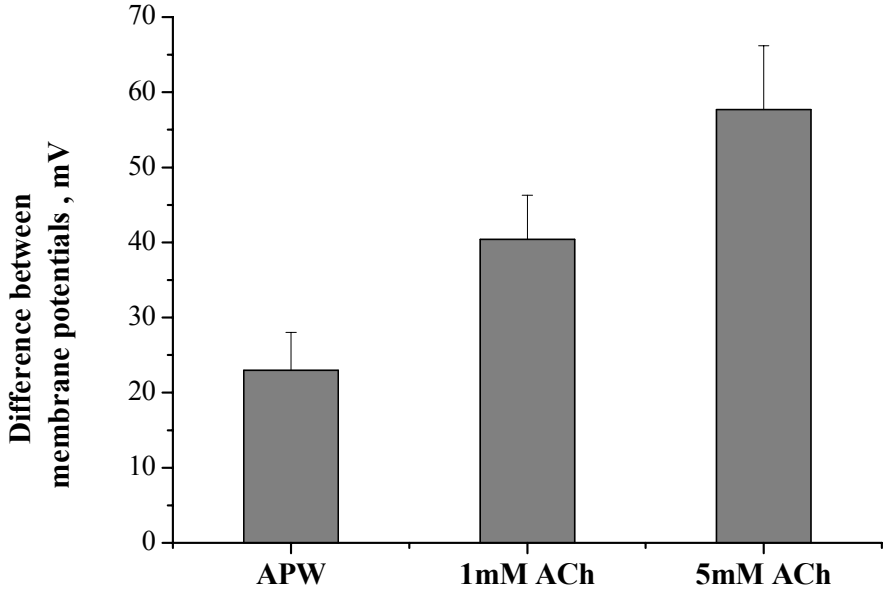

Figure 3.4. The effect of ACh on differences between membrane potentials before and 5 min after stimulation.

Since ACh modulates slow repolarization we decided to investigate the effect of ACh on membrane potential dynamics after repetitively triggered APs.

3.4. 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 (Shepherd et al., 2008), 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), so we decided to evoke repetitive excitation of cells resulting in elevation of membrane potential. We analyzed repetitively triggered APs, their reproducibility, shape and membrane potential dynamics after AP induction in APW and ACh solutions and noticed that ACh caused changes in membrane potential dynamics 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 (lines in **Figure 3.5**).

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

Where y_0 is membrane potential before stimulation, A_1 describes range of membrane potential dynamics and t_1- time constant (**Figure 3.5**).

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 in APW reached plateau after the fifth AP, 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 (**Figure 3.5**).

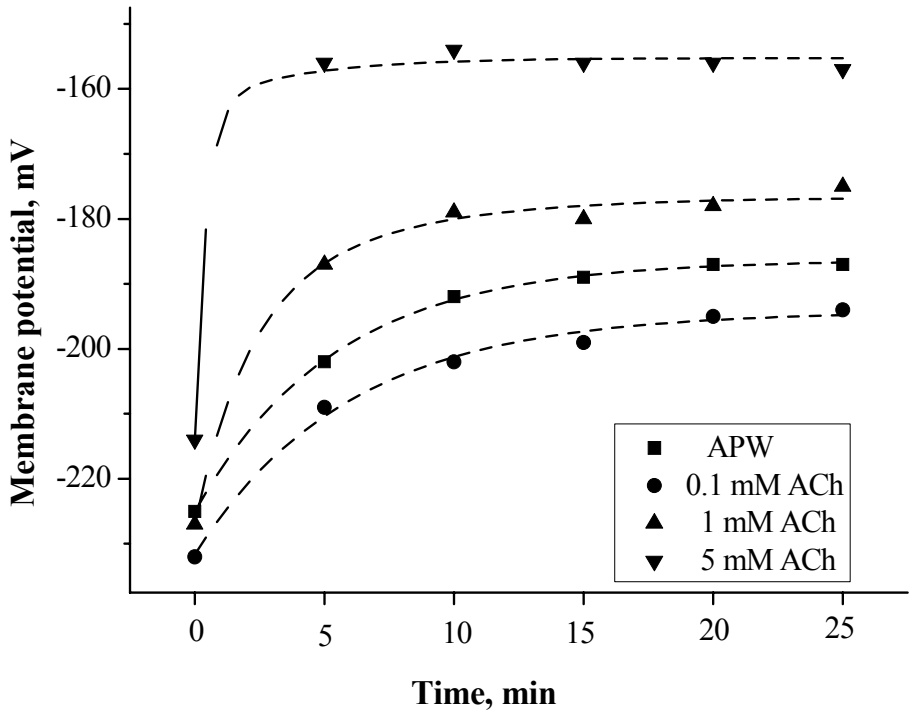

Figure 3.5. Effect of ACh on dynamics of slow repolarization of average membrane potential after repetitively triggered APs (n=6). Repetitive stimuli were applied every 5 min. (see **Figure 3.2**). Dashed lines - fitted exponential model curves. Slow repolarization was evaluated as the value of membrane potential at the moment of stimulus application, except the value at time 0 min which was resting membrane potential.

3.5 The effect of acetylcholine on membrane potentials after inhibition of proton pump

The membrane potential of characean cells is composed of two components- passive diffusion potential and active potential generated by an electrogenic proton pump. It is possible that reason of membrane potential depolarization after ACh application could be decrease in conductance of H^+-ATPase. In order to investigate the effect of ACh on membrane potential component determined by passive diffusion, we pharmacologically inhibited H^+-ATPase. It was found that 50 μM concentration of DCCD is sufficient to give complete and irreversible inhibition of proton pumping whilst

leaving the passive diffusion of K^+ ions unaffected (Sokolik & Yurin,1986). Irreversible depolarization of membrane potential and decrease of membrane conductance we used as indicators of proton pump inhibition in *Nitellopsis obtusa* cells in our experiments. Resting potential diminished to -145 ± 2,6 mV from -230,3 ± 4,6 mV and plasma membrane resistance increased from 26,9 ± 1,9 kΩ/cm^2 to 91 ± 3,6 kΩ/cm^2 after DCCD application.

It was found that ACh strengthened membrane potential depolarization, but decreased membrane resistance **(Figure 3.6)**. The effect of ACh on membrane potential and resistance was reversible. In many cases (8 of 12) DCCD and ACh application led to a spontaneous activity of the cell (**Figure 3.7**).

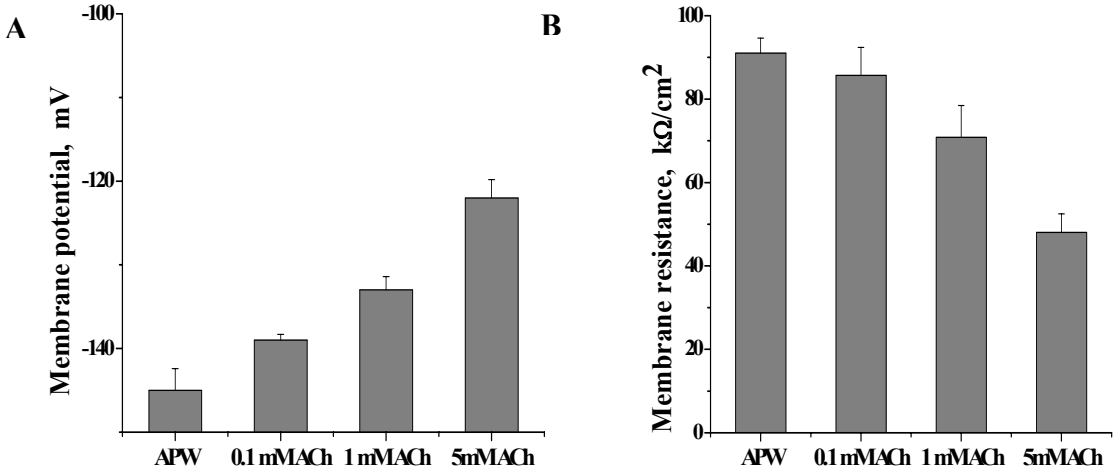

Figure 3.6. The effect of ACh on membrane potential (A) and resistance (B) after the inhibition of proton pump by 50 μM concentration of DCCD.

It was observed, that ACh determined increase of AP duration irrespective of whether AP was spontaneous or electrically evoked**.** When cells after washing were repetitively affected by ACh (3 cells), we saw the same effect. We evaluated width of AP at 0 and -70 mV and found expansion of AP as concentration of ACh increased **(Table 3.2)**. This means ACh prolongs AP repolarization in the cells which membrane potential is determined by diffusion and ion channel activity **(Figure 3.8)**.

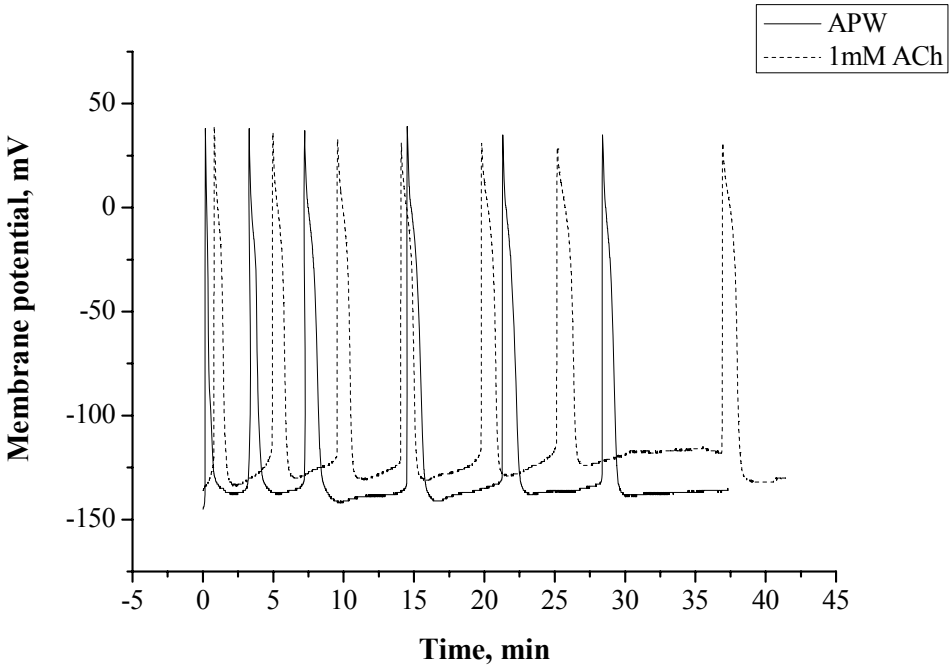

Figure 3.7. The effect of ACh on spontaneous activity of *Nitellopsis obtusa* cells after H^+-ATPase inhibition (typical example). The cells were treated for one hour with 50 μM DCCD solution to inhibit proton pump. After that cells were washed out for one hour with APW and finally treated with ACh for one hour. Figure shows last spontaneous AP in APW solution (black lines) and spontaneous activity straight after 1mM ACh application (dotted line).

Table 3.2. Increment of the second AP duration (times) determined by application of 1 and 5mM ACh solution after blocking of metabolic component of membrane potential with 50 μM DCCD solution. Duration of AP was measured at 0 mV and 70 mV.

Potential / Solution	Change of AP duration (times)	
	0 mV	70 mV
1 mM	1,9 ± 0,5	1,7 ± 0,2
5 mM	2,6 ± 0,5	2,7 ± 0,4

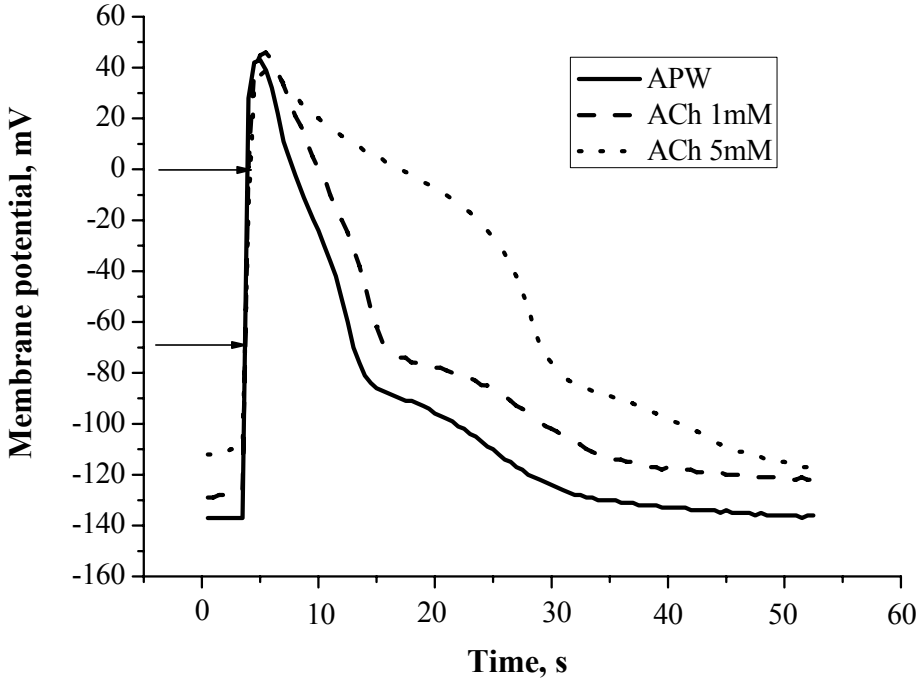

Figure 3.8. Typical example of ACh effect on the second AP after blocking of metabolic component of membrane potential with 50 μM DCCD solution. Arrows indicate levels of AP at which duration of AP was measured.

3.6 The effect of acetylcholine on action potentials of cells in naturally stable K^+ state

In the ordinary conditions viable *Nitellopsis obtusa* cells seldom can be found in stable K^+ state (RP ~ -160 mV) for a long time. Very often we can observe diurnal activation of H^+ATP-ase and RP hyperpolarization to -220 mV and more negative values. We found only 5 cells from 159 investigated in naturally stable K^+ state and examined effect of ACh on their membrane potentials. RP of such cells in APW solution was 161,2 ± 3,1 mV. Application of 5 mM ACh determined gradual depolarization of the cell and spontaneous activity after membrane potential reached -122,5 ± 2,8 mV. We compared the second AP in APW and 5 mM ACh solutions and found significant increase in duration of AP repolarization after ACh application (**Figure 3.9 A**). Width of AP evaluated at 0 and -70 mV reveals prolongation of AP repolarization after application of 5mM ACh by 1,5 ± 0,2 and 2,1 ± 0,3 times respectively (**Figure 3.9 A**).

To evaluate velocity of repolarization, we differentiated 50 s periods starting from the peak of AP. We found that the highest rate of repolarization declined from 24,8± 0,8 mV/s in APW solution to 14,4 ± 1 mV/s after application of 5 mM ACh. This effect of ACh was reversible.

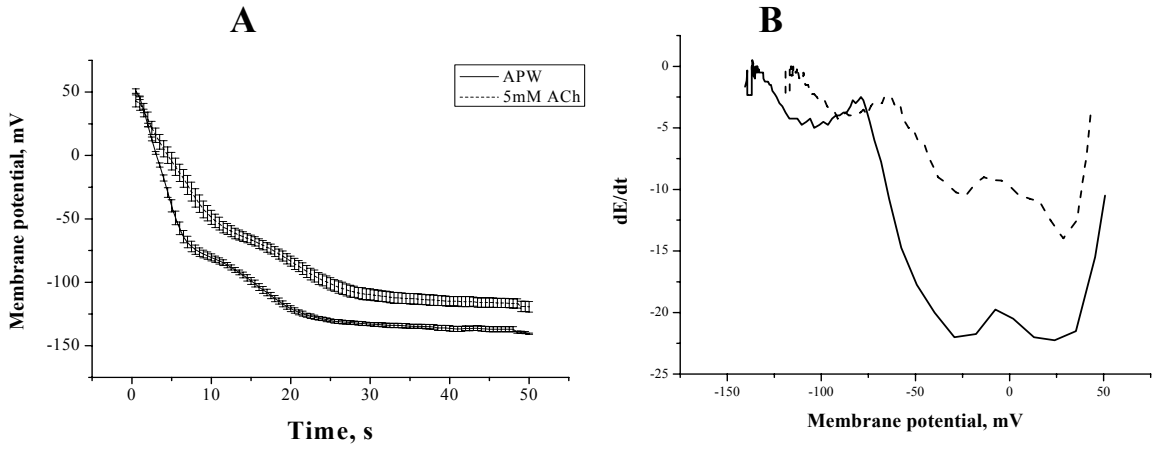

Figure 3.9. Averaged repolarization of the second AP in APW (black line) and in 5 mM ACh (dotted line) solutions (n=5) lasting for 50 s. Vertical bars – confidence intervals (**A)**. Average phase plane of repolarization dynamics of the second AP in APW (solid line) and 5 mM ACh (dashed line) (n=5). 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 (**B)**.

It is generally accepted that certain ion processes start at a particular value of membrane voltage (Yurin et al., 1991). Therefore, it was interesting to investigate if the highest repolarization rate after ACh application occurs at the same voltage in different solutions. 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 3.9 B**). It was found that ACh reduced rate of AP repolarization in the range of all investigated potentials, especially at -25 mV (**Figure 3.9 B**).

3.7. The effect of acetylcholine on action potentials of the cells 16 hours treated in 1mM ACh solution

In order to investigate if time of ACh application amplifies the effect of ACh we kept *Nitellopsis obtusa* cells in 1 mM ACh solution for 16 hours. The same pattern of the ACh effect - increased time of application strengthened depolarizing effect on membrane

potential and prolonged AP repolarization - was found in all investigated cells (**Figure 3.10**).

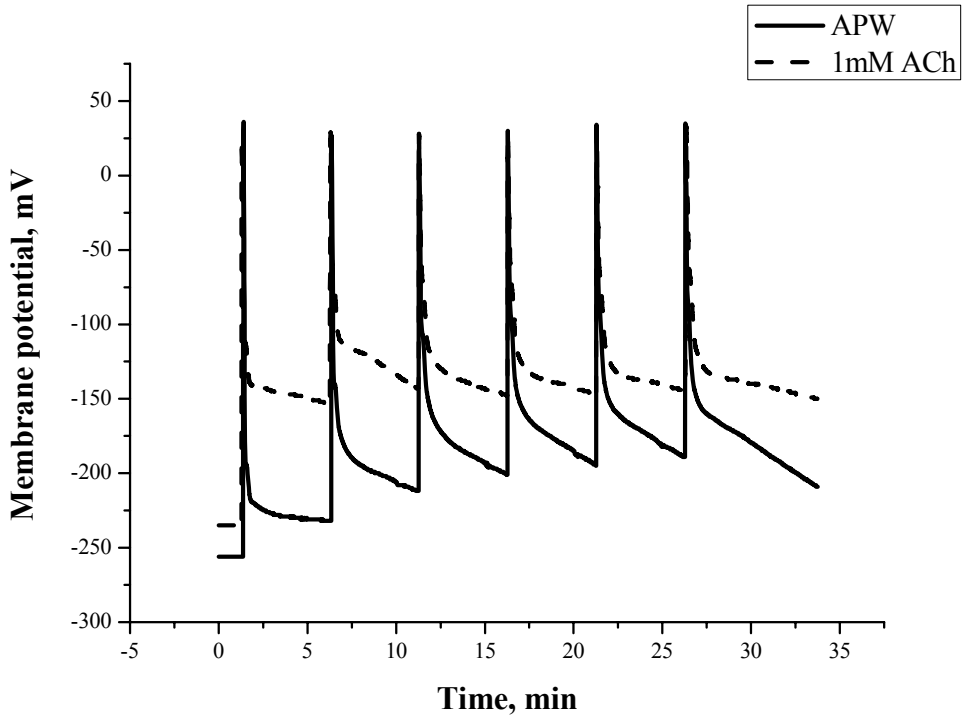

Figure 3.10. Typical example of the effect of 16 hour lasting application of 1 mM ACh on dynamics of repetitively triggered AP (n=6)

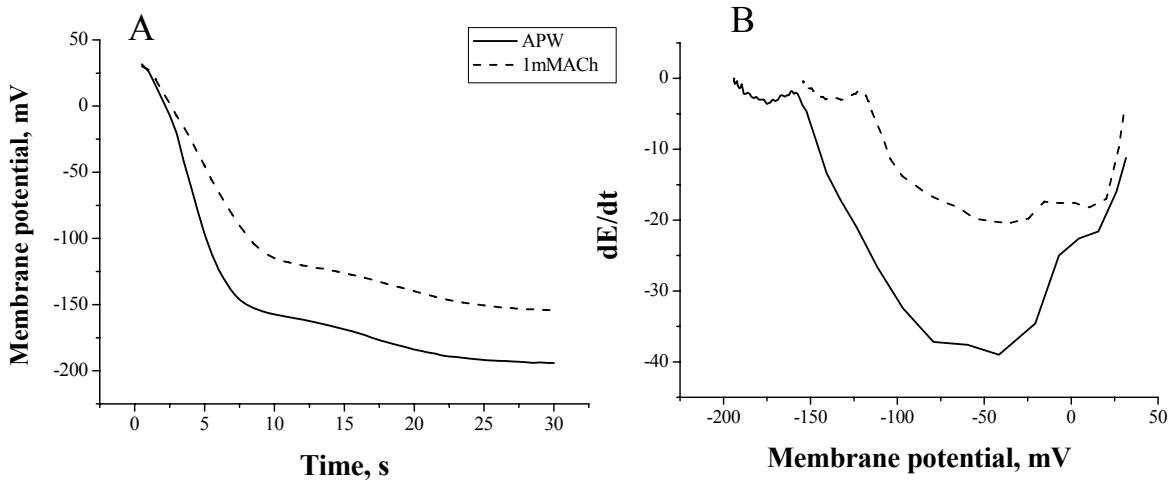

Figure 3.11. Averaged 50 s lasting repolarization of the second AP in APW (black line) and after 16 hours application of 1 mM ACh (dashed line) solutions (A) and average phase plane of repolarization dynamics of the second AP. 50 s period starting from the peak of AP was differentiated to evaluate velocity of repolarization. The dE/dt is plotted against the membrane voltage (B). (n=6)

Statistical analysis revealed that dynamics of repetitively triggered AP was similar when cells were treated 1 hour with 5 mM ACh and 16 hours with 1 mM ACh ($p>0,2$, $n=6$).

It has been found, that application of 1mM ACh lasting 16 h modulates repolarization process by increasing its duration (**Figure 3.11 A**). Maximal repolarization velocity for treated cells was 1,91 ± 0,1 times less than in APW solution after washing **(Figure 3.11 B).**

3.8. The combined effect of organophosphates and acetylcholine on membrane permeability of *Nitella flexilis* cells

It is generally agreed that activity of AChE is inhibited by organophosphates. So we decided to investigate the effect of 10μM insecticide dimetoat (DIM) on the permeability of *Nitella flexilis* plasma membranes. Application of ACh decreased membrane resistance of *Nitella flexilis* cells from 22,28 ± 2,2 kΩ/cm^2 in APW solution to 17,64 ± 2 kΩ/cm^2 in 1mM ACh and to 11,73 ± 2,3 kΩ/cm^2 in 5mM ACh solutions. DIM *per se* increased membrane permeability, however we found DIM strengthen impact of 5mM ACh on increase of membrane permeability **(Figure 3.12)**. Membrane permeability increased 1,9 times after 5mM ACh application and 2,7 times after combined application of 5 mM ACh and DIM. This effect was reversible.

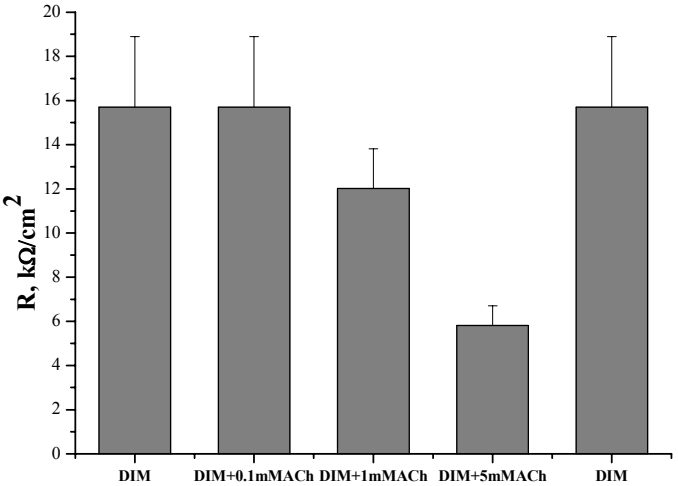

Figure 3.12. The combined effect of dimetoat and acetylcholine on membrane resistance (R, kΩ/cm^2).

3.9. The effect of cadmium on membrane electrogenesis in *Nitellopsis obtusa* cells

It has been shown cadmium significantly inhibits AChE activity *in vitro* so we decided to examine Cd^{2+} as a possible inhibitor of AChE *in vivo.* We found that cadmium at 100 μM and higher concentrations after 30 min application caused strong depolarization of the plasma membrane (PM) potential in 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 3.13)**. 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.

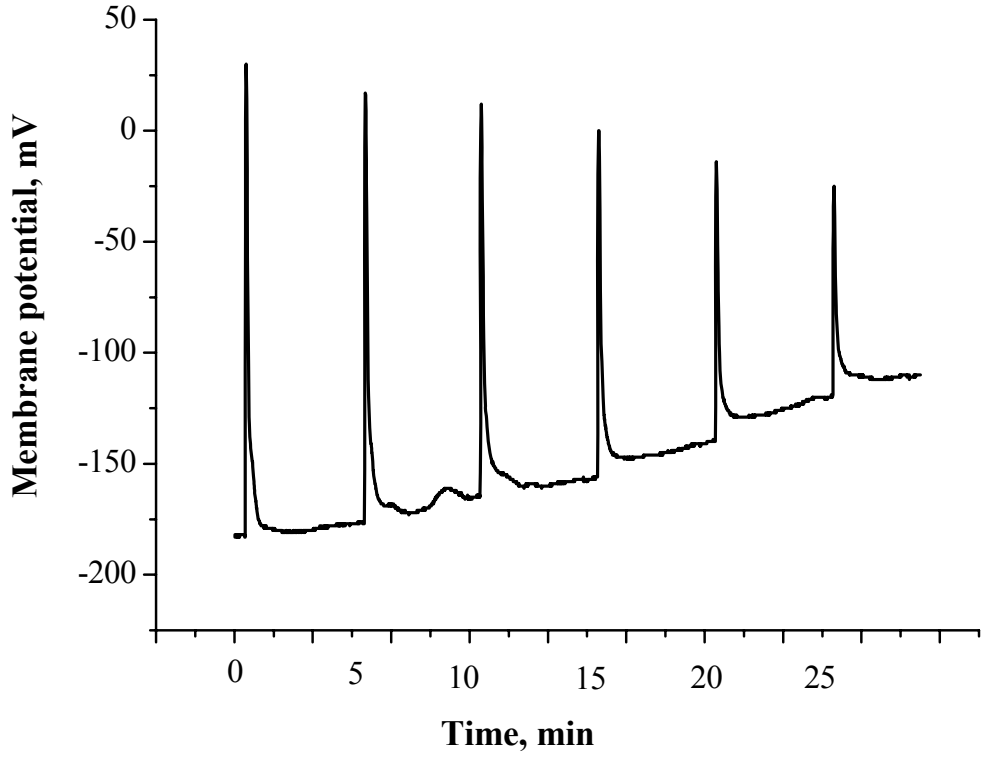

Figure 3.13. Typical example of membrane potential dynamics after repetitively triggered APs. The cell was treated with 100 μM Cd^{2+} solution for 20 min. before stimulation.

It is considered that strong irreversible depolarization of membrane potential is indicator of Cd^{2+} toxicity (Llamas et al., 2000). 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.*

3.10. The combined effect of cadmium and acetylcholine on membrane potential.

We analyzed 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 3.14)**. 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, though 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 repolarization of membrane potential after the first stimulus **(Figure 3.14)**.

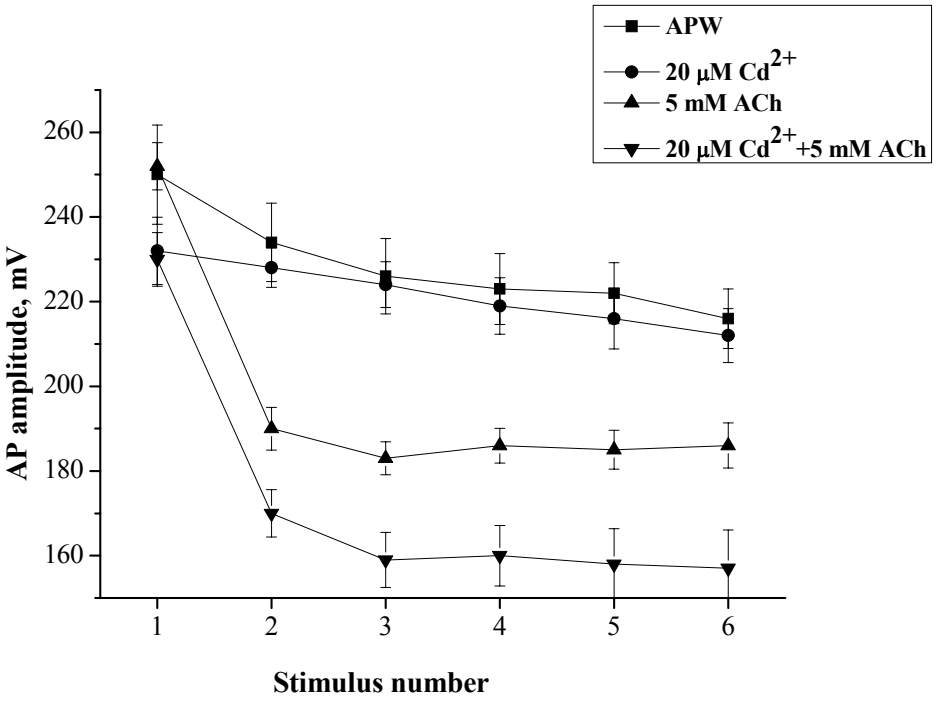

Figure 3.14. Average amplitude reduction of repetitively triggered APs after treatment with 20 μM Cd^{2+}, 5 mM ACh or 5 mM ACh applied together with 20 μM Cd^{2+} solutions (n=8). Time between stimuli is 5 min. Amplitude was evaluated as difference of membrane potential value before the moment of stimulus application and AP peak.

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 3.15)**. 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 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>0,1) between effect of ACh and ACh plus Cd^{2+} solutions **(Figure 3.16).**

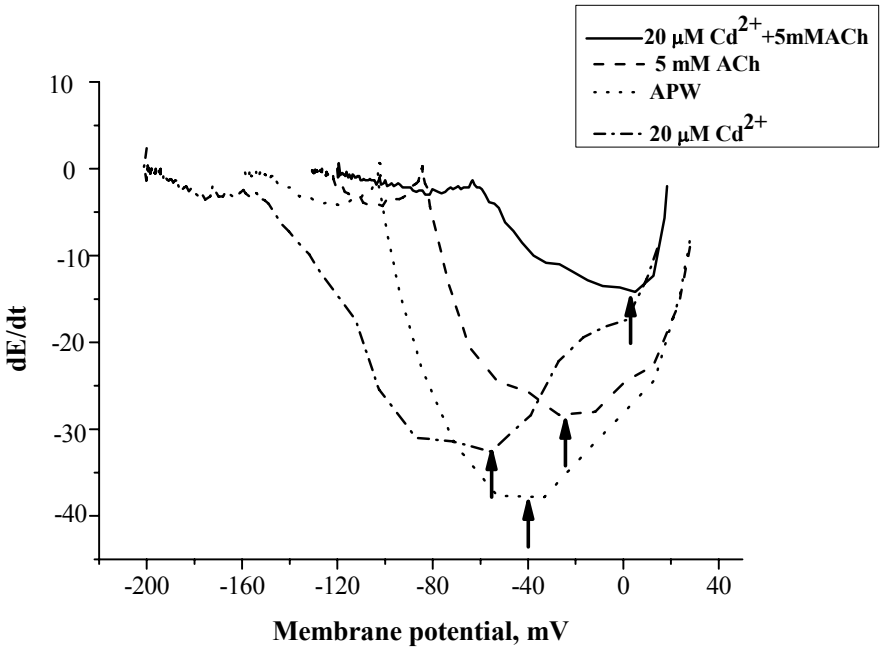

Figure 3.15. Average phase plane of the second AP repolarization dynamics after application of 20 μM Cd^{2+}, 5 mM ACh or 5 mM ACh applied together with 20 μM Cd^{2+} (n=8). 50 s period starting from the peak of AP was differentiated to evaluate velocity of repolarization. The dE/dt is plotted against the membrane voltage.

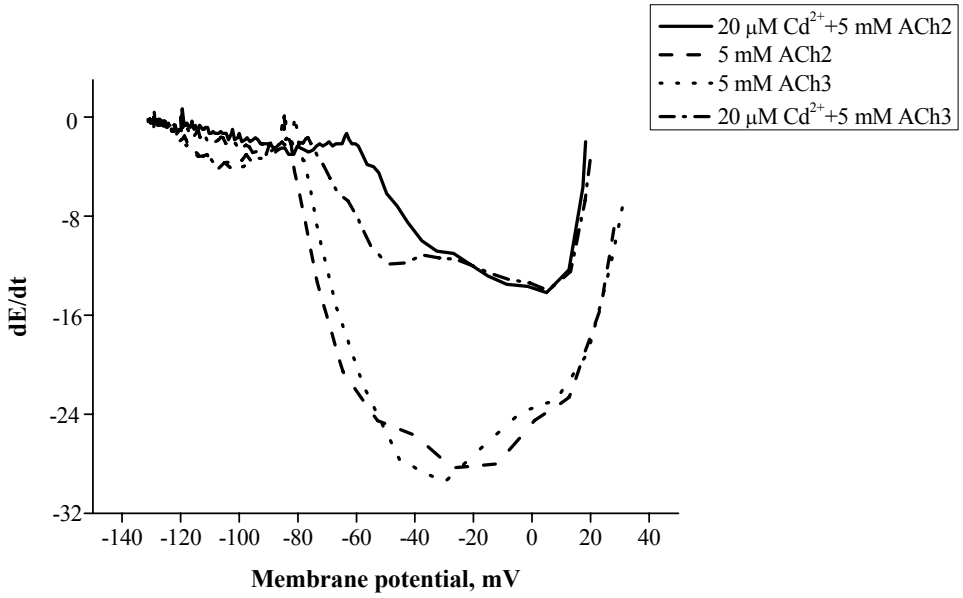

Figure 3.16. Average phase plane of repolarization of the second (5 mM ACh 2 and 5 mM ACh+20 μM Cd^{2+} 2) and the third (5 mM ACh 3 and 5 mM ACh+20 μM Cd^{2+} 3) AP dynamics. 50 s period starting from the peak of AP was differentiated to evaluate velocity of repolarization. dE/dt was plotted against the voltage (n=8).

3.11. The combined effect of neostigmine bromide and acetylcholine on membrane potentials in *Nitellopsis obtusa* cells

In order to test influence of acetilcholinesterase inhibitors on ACh induced membrane potential changes in Characeaen cells we examined specific inhibitor of AChE neostigmine bromide (NB). 30 μM NB solution was used for AChE inhibition in *Nitellopsis obtusa* cells. We found that NB not affected membrane potential *per se* ($p<0,22$). However when after 1 hour application of 30 μM NB cell was treated by combined solution of 0,03mM NB and 5mM ACh it was observed enhanced depolarizing effect of ACh. Furthermore, 7 out of investigated 13 cells after the first stimulus generated dual action potentials – the second spontaneous AP rised while repolarisation was not completed (**Figure 3.17**).

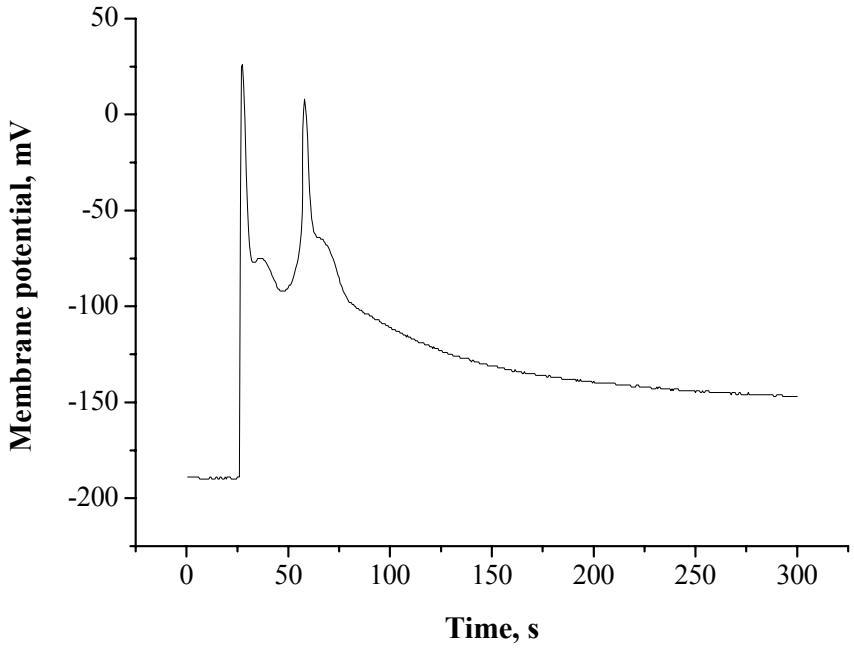

Figure 3.17. Typical example of dual action potential electrically evoked after AChE inhibition and 5 mM ACh application.

The amplitude and time of generation of the second spike was different in different cells. Despite this it was noticed that cells could be divided into two groups depending on AP repolarisation. There were no dual AP if membrane potential after 50 s lasting repolarisation in APW (and NB) solutions reached 201 ± 6,1 mV, (n=6) (**Figure**

3.18 B). But if membrane potential after 50 s lasting repolarisation in APW (and NB) solutions reached 170 ± 6,7 mV (n=7) combined solution of 0,03 mM NB and 5 mM ACh caused dual AP of various forms and amplitudes (**Figure 3.17, Figure 3.18 A)**.

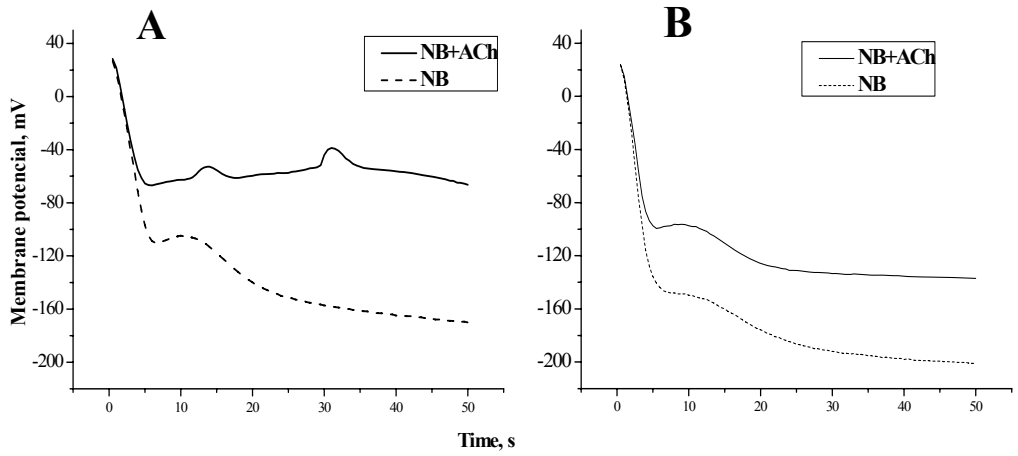

Figure 3.18. The averaged repolarization of the first AP in 0,03 mM NB and 0,03 mM NB+5mM ACh solutions. A - membrane potential after 50 s lasting repolarization of the first AP in NB solution reached 170 ± 6,7 mV (n=7) and dual action potentials were observed. B – membrane potential after 50 s lasting repolarization of the first AP in NB solution reached 201 ± 6,1 mV, (n=6) and dual action potentials were not observed.

The effect of NB and combined effect of 0,03 mM NB + 5 mM ACh on dynamics of AP was analyzed. There were no statistically significant differences ($p<0,3$) between the amplitudes of repetitively triggered APs in APW and NB solutions (**Figure 3.19**). There was decrease of all amplitudes and distinct effect on the second AP after application of 0,03 mM NB + 5 mM ACh (**Figure 3.19**). Unpaired t-test revealed significant differences between the amplitude of all AP in 5 mM ACh and 0,03 mM NB + 5 mM ACh ($p>0,01$, n=10) solutions. We found that 30 μM NB like 20 μM Cd^{2+} strengthens the depolarizing effect of acetylcholine on membrane potential repolarisation after the first stimulus, and there were no significant differences ($p<0,13$) between membrane potential repolarisation in these solutions.

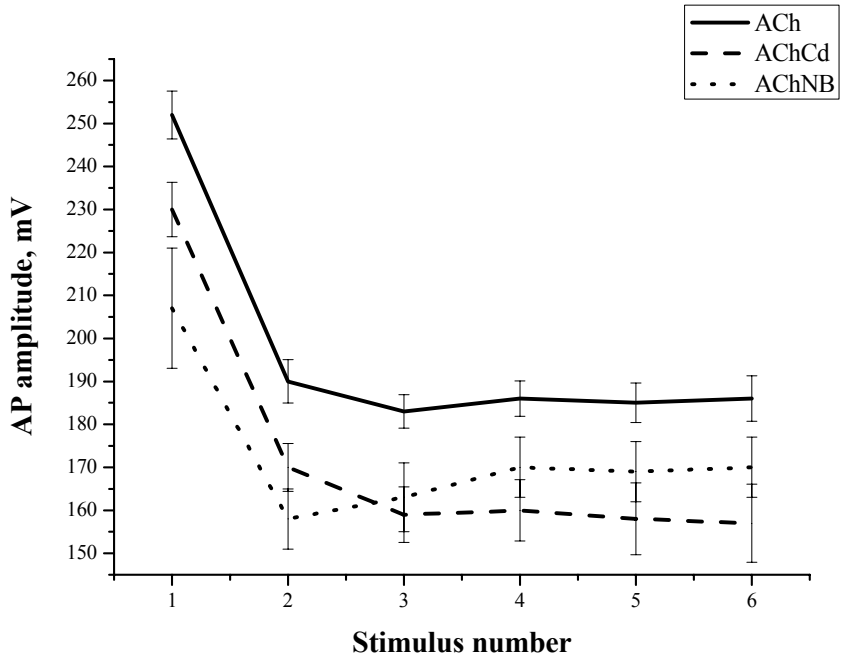

Figure 3.19. Average amplitude reduction of repetitively triggered APs in 5 mM ACh, 20 μM Cd^{2+} + 5 mM ACh and 30 μM NB+5mM ACh solutions (n=10). Time between stimuli is 5 min. Amplitude was evaluated as difference of membrane potential value before the moment of stimulus application and AP peak.

3.12. The effect of nicotine on membrane potentials in *Nitellopsis obtusa* cells

Investigating the influence of ACh agonist nicotine on electrical responses of *Nitellopsis obtusa* cells the same effect was observed as by application of ACh, however nicotine was effective in smaller concentrations as compared to ACh. 0,1 mM nicotine caused strong depolarization of membrane potential, which started immediately after solution change. Depolarizing effect of nicotine depends on energetic state of the cell and resting potential in control conditions. Cells with very negative (> -230 mV) RP values expressed fast repolarization - depolarizing by 52 ± 7 mV (n=5) in 30 min after 0,1 mM nicotine application. Changes in AP amplitude and velocity of repolarization were observed after nicotine application (**Figure 3.20**). Difference between slow repolarisation values after the first AP in APW and 0,1 mM nicotine solution was 82 ± 9 mV (n=5). We assessed the effect of nicotine on the second AP the same way as in ACh investigation and found decrement of AP amplitude

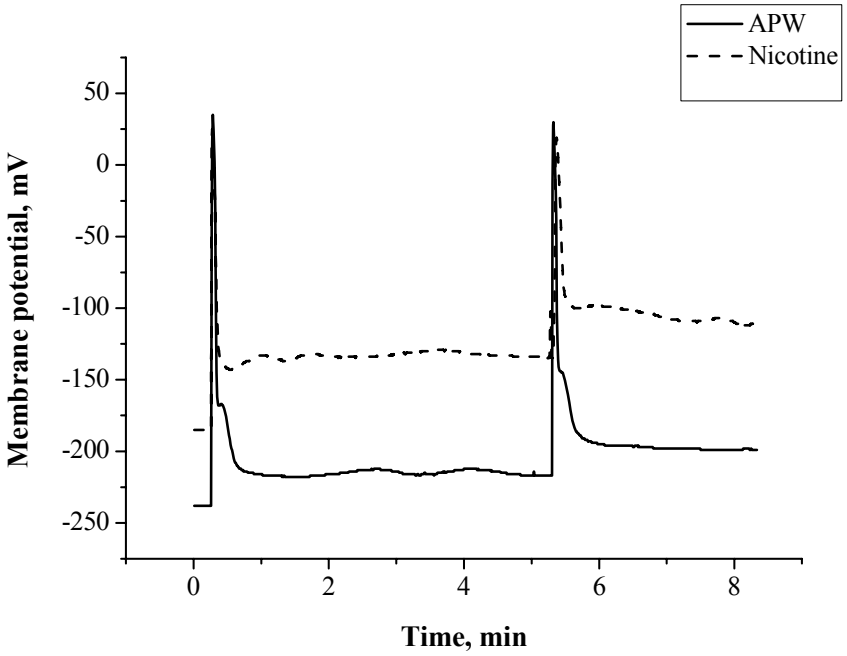

Figure 3.20. The effect of 0,1 mM nicotine on membrane potential dynamics of *Nitellopsis obtusa* cells with very negative potential (> -230 mV) and fast repolarization. The effect of nicotine lasted 30 min before stimulation and during stimulation time.

from 226 ± 11 mV to 118 ± 14 mV (n=5). The highest value of fast repolarization rate after nicotine application decreased by 4 ± 0,6 times (n=5) (**Figure 3.21**).

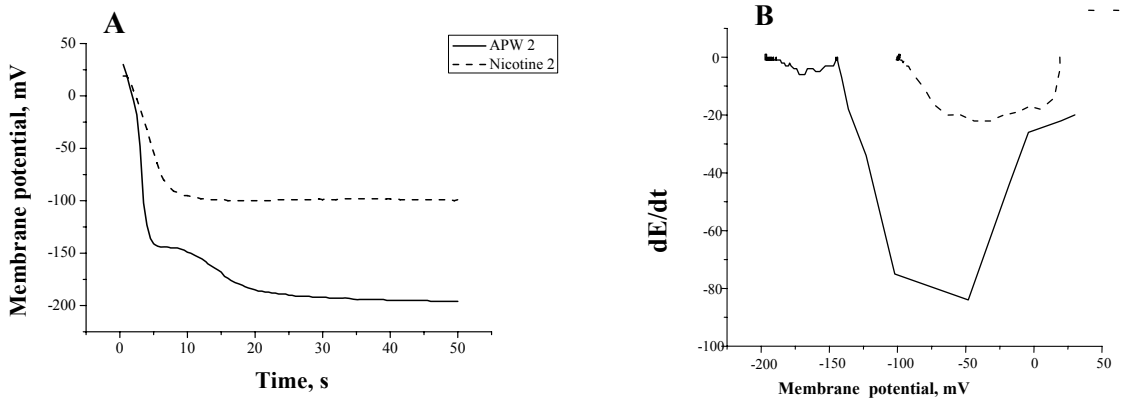

Figure 3.21. The average repolarization of the second AP after 0,1 mM nicotine treatment lasting 30 min (A) and phase plane of repolarization (B), (n=5).

0,1 mM nicotine caused strong depolarization and spontaneous activity of the cell if membrane potential of the cell was about -220 mV (n=5). In some cases membrane potential depolarized up to -60 mV. But this effect was reversible despite of strong depolarization. Cells with membrane potential more negative than –100 mV after nicotine application fully restored control value in hour after washing out (n= 5). Cells with membrane potential depolarized up to more than –100 mV after nicotine application, reached -162 ± 17 mV. value in hour after washing out (n= 5).

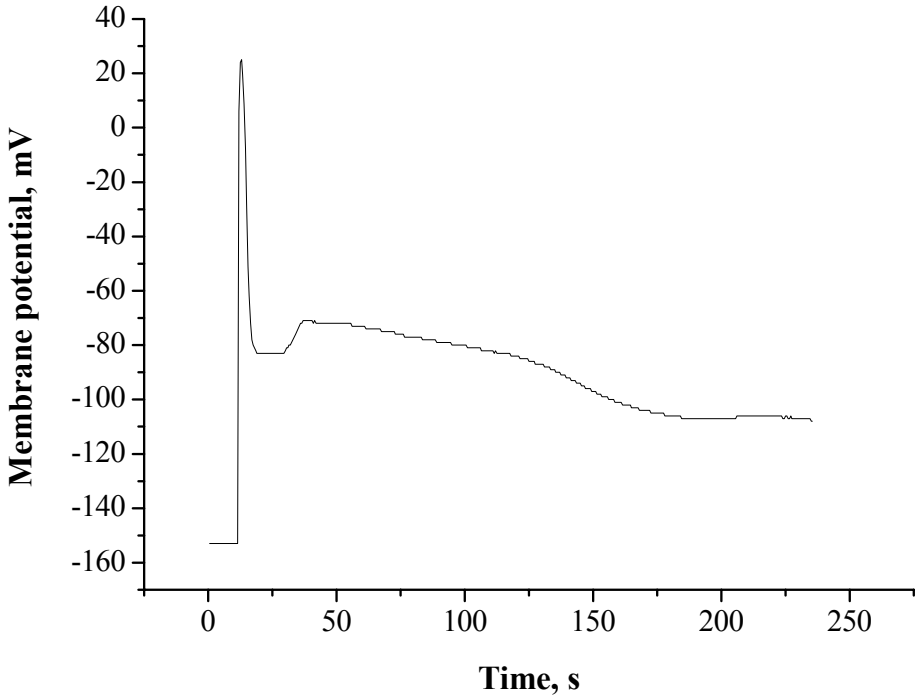

Figure 3.22. Typical example of dual action potential electrically evoked by application 0,05 mM nicotine.

0,05 mM nicotine caused cells (n=5) MP depolarization and dual action potentials like in the case of application of neostigmine bromide and ACh (**Figure 3.22**).

3.13. The effect of acetylcholine on excitability of *Nitellopsis obtusa* cells

Investigating influence of ACh on action potentials of *Nitellopsis obtusa* cells we noticed increase in spontaneous activity which was caused not only by depolarizing effect of ACh but increase in excitability of the cell too. In the ordinary conditions

excitation threshold of the *Nitellopsis obtusa* cells is 90 ± 10 mV. Voltage clamp experiments revealed that acetylcholine reduced the excitation threshold in all investigated cells ($p < 0,001$, n=7, **Figure 3.23**). In many cases rice of Cl^- current was noticed at -80 mV in APW solutions whereas in 5mM ACh solution at -120 mV. In all cases the effect of acetylcholine was reversible. Application of 0,05 mM of nicotine increased negativity of the excitation threshold by 20 mV (n=5).

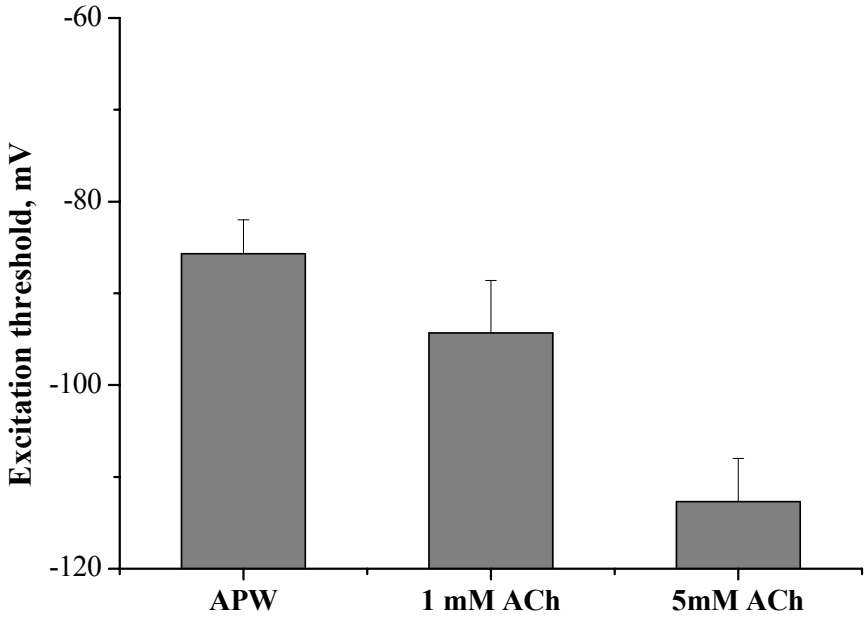

Figure 3.23. Average excitation threshold in 1 mM and 5 mM ACh solutions.

It was noticed the influence of ACh on the time of rice of Cl^- current. In the control solution activation of Cl^- current appears after 212 ± 12 ms from stimulation onset. 1 mM ACh reversible reduced latency of Cl^- current 58 ± 10 ms if compare to APW solution ($p<0,00001$, n=7). In the 5mM ACh solution activation of Cl^- current reversible appears after 148 ± 9 ms from stimulation onset ($p<0,00001$, n=7). Diferencies between 1 mM and 5mM ACh was not significant ($p>0,05$, n=7). So increase of excitability of *Nitellopsis obtusa* cells after ACh application due to decrement of time to excite the cell and more negative excitation threshold.

3.14. The effect of acetylcholine on Cl^- current of *Nitellopsis obtusa* cells

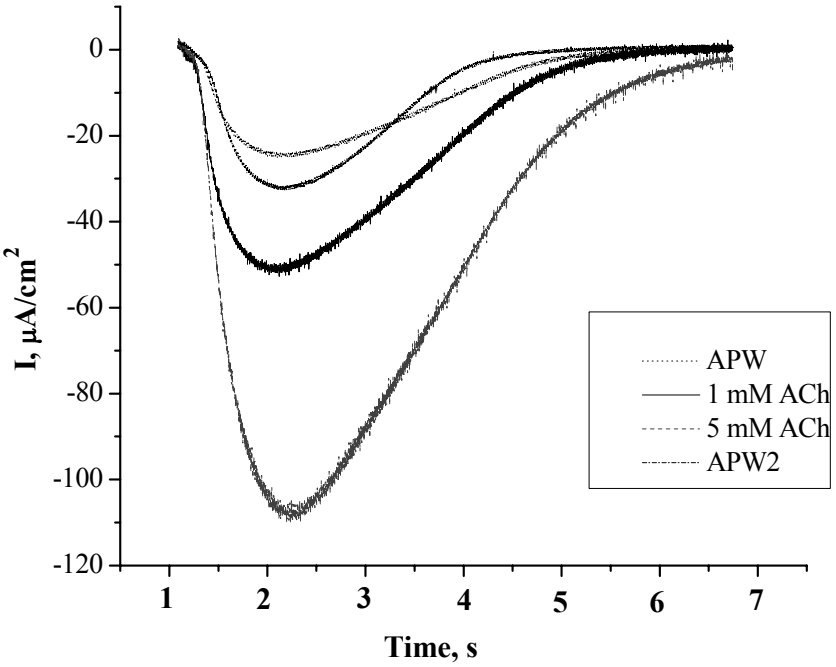

Figure 3.24. Typical example of Cl^- current generation above excitation threshold registered at -80 mV.

It has been found that ACh caused increase of Cl^- current (**Figure 3.24).** This increment was found at all tested voltage ranges (**Figure 3.25).**1 mM ACh increased Cl^- current on the average by 1,6 ± 0,05 times (*e.g.* current increased by 16,2 ± 6,6 μA/cm^2 at – 60 mV voltage). 5mM Ach increased Cl^- current on the average by 2,7 times (*e.g.* current increased by 51,3 ± 6,7 μA/cm^2 at – 60 mV voltage) comparing with measured current amplitude in APW solution.

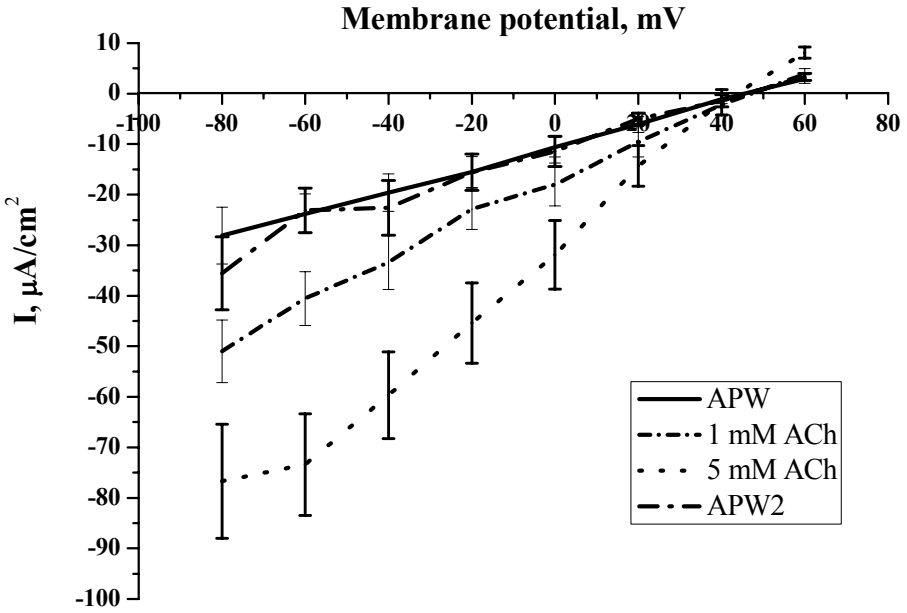

Figure 3.25. Dependence of the impact of acetylcholine on Cl^- current of *Nitellopsis obtusa* cell. Averaged current - voltage relations. Families of current curves after excitation were obtained by the sequence of single pulses of 20 mV applied every 5 min.

It was noticed that ACh increased not only the amplitude of Cl^- current but also the time of Cl^- channels presence in the open state ($p<0{,}001$, $n=7$, **Figure 3.26**).

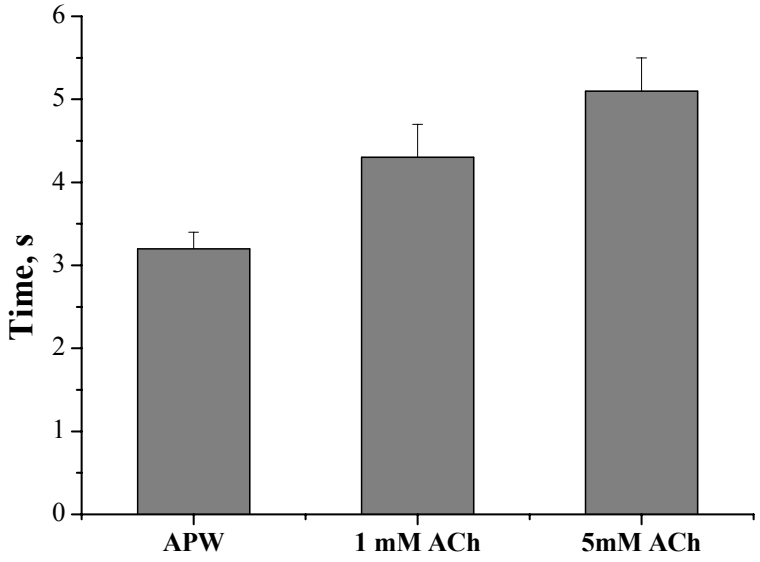

Figure 3.26. Dependence of duration of Cl^- current inactivation on ACh concentration registered at -60 mV voltage.

The increment of duration of Cl^- current inactivation at other voltages was analogous: 1 mM ACh increased duration of Cl^- current inactivation on the average by 0,9 ± 0,3 s (25%.), and 5 mM ACh – 1,6 ± 0,5 s (46 %.).

1mM ACh and 5 mM ACh shifted Cl^- current reversal potential in negative direction fractionally but statistically significant ($p<0,001$). Reversal potential of Cl^- current was 49,4 ± 2,4 mV in APW solution, 45,2 ± 2,5 mV in 1mM ACh, and 43,2 ± 2,5 mV. in 5 mM ACh.

3.15. The effect of acetylcholine on Ca^{2+} current in *Nitellopsis obtusa* cells

Under physiological conditions beyond a threshold voltage in voltage clamped characeaen cells activation of transient currents includes calcium i_{Ca} and chloride i_{Cl} components. They are different in amplitude and time of activation. The i_{Ca} arising 100 ms after stimulation was best separated at 20 mV voltage. We measured this current and found increase of amplitude value after ACh application **(Figure 3.27).**

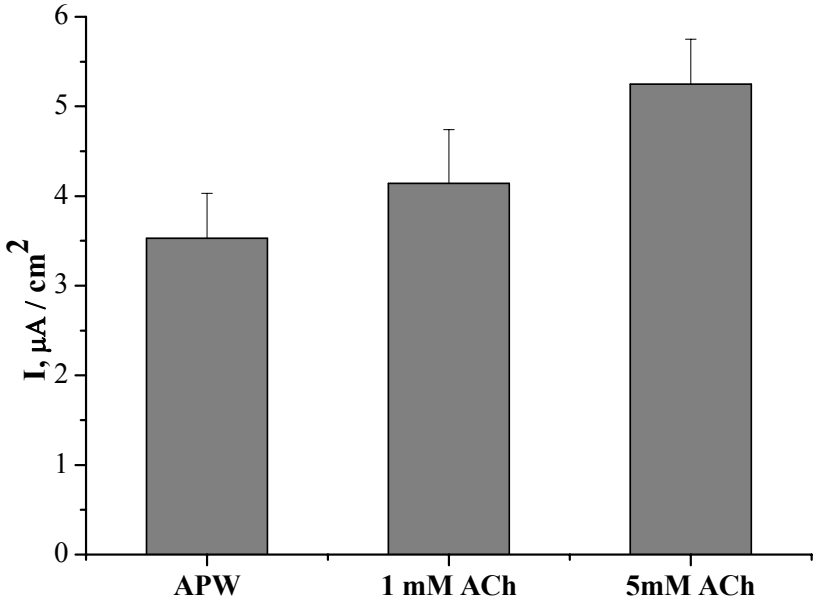

Figure 3.27. The impact of acetylcholine on Ca^{2+} current registered at 20 mV voltage.

It was established that 5 mM ACh increased the amplitude of Ca^{2+} current by 33 ± 12% ($p=0,025$, $n=7$).

3.16. The effect of acetylcholine on K^+ channels in *Nitella flexilis* cells

Plant K^+ channels include two large classes of outwardly and inwardly rectifying K^+ channels. They are activated at different voltages: K_{in}^+ are active after membrane potential hiperpolarization, K_{out}^+ are active after membrane potential depolarization. So we investigated the effect of ACh on outward and inward K^+ currents separately. It was found that ACh increased conductance of both types of K^+ channels.

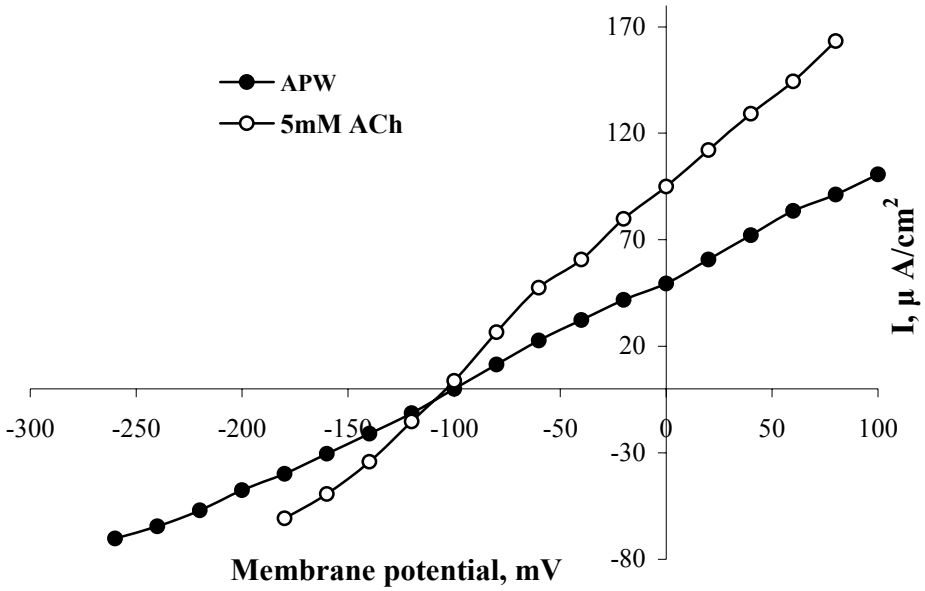

Figure 3.28. Typical example of voltage - current relations of K_{out}^+ channels activated by depolarization in APW and 5mM ACh solutions. Membrane potential was clamped at -40 mV. Short (30 ms) rectangular depolarization or hyperpolarization current pulses were injected every 20 mV step.

Conductance of K_{out}^+ channels after 5 mM ACh application increased by 57,5 ± 5,7%. K^+ current value was assessed at 40 mV voltage. We found that outward current value significantly ($p < 0{,}001$, n=6) increased by 64 ± 5,5% after 5 mM ACh application. Reversal potential slightly (on average 5 mV) shifted towards the negative direction (**Figure 3.28).**

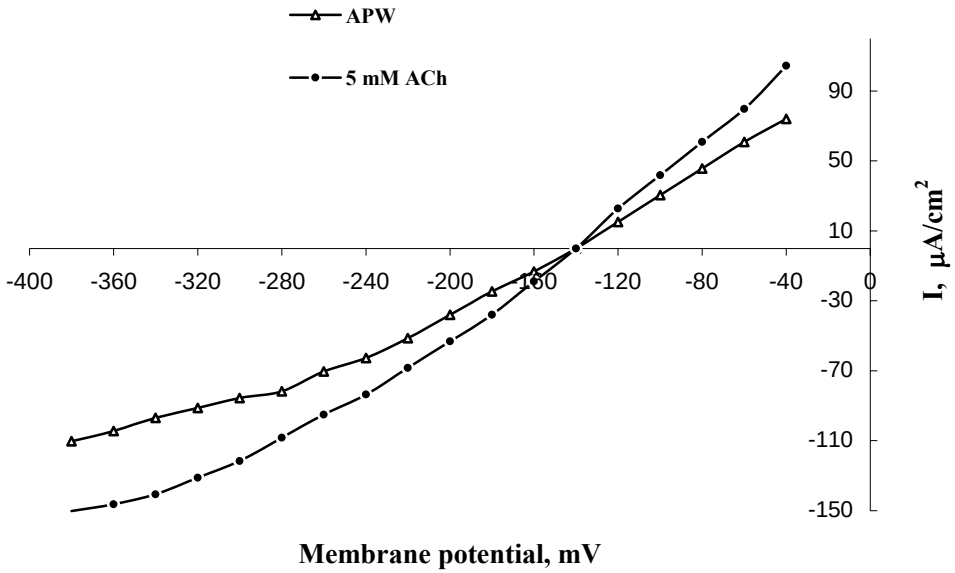

Figure 3.29. Typical example of voltage - current relations of K_{in}^+ channels activated by hyperpolarization in APW and 5mM ACh solutions. Membrane potential was clamped at -160 mV. Short (30 ms) rectangular hyperpolarization or depolarization current pulses were injected every 20 mV step.

Conductance of K_{in}^+ channels after 5mM ACh application increased by 53,5 ± 3,4%. K^+ current value was assessed at -340 mV voltage. We found that inward current significantly ($p<0,002$, $n=6$) increased by 41 ± 6,8% after 5mM ACh application. Reversal potential remained the same (**Figure 3.29).**

3.17. The effect of acetylcholine on H^+-ATPase activation

Characeaen *Nitella flexilis* internodal cells provided the useful experimental material in proton pump investigations because of possibility to modulate activity of H^+-ATPase by light. In those cells, where photoinduction reaction was elicited, we noticed effect of acetylcholine. Application of 5 mM ACh hyperpolarized membrane potential by 14 ± 3 mV.

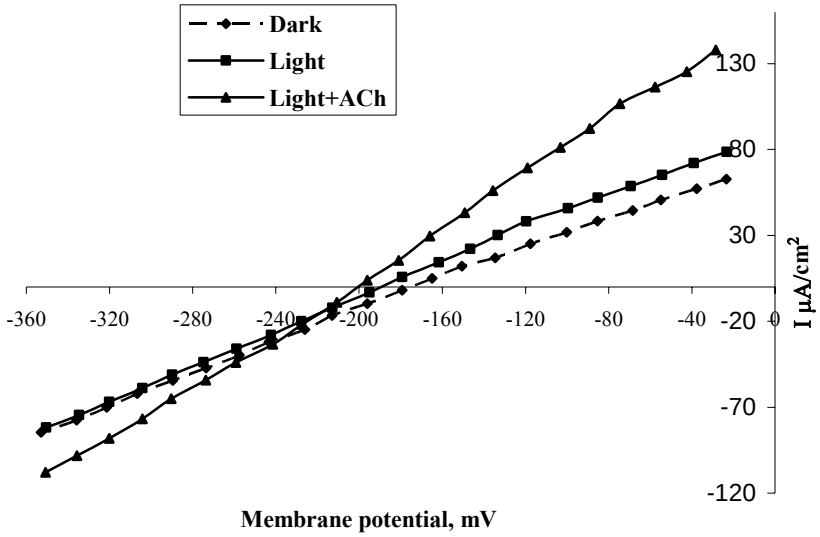

Figure 3.30. Typical example of voltage - current relations of H^+-ATPase activation by light in APW and 5 mM ACh solutions. Membrane potential was clamped at -160 mV. Short (30 ms) rectangular hyperpolarization or depolarization current pulses were injected every 20 mV step. Voltage - current relations were taken in dark, light conditions and after ACh application in the light.

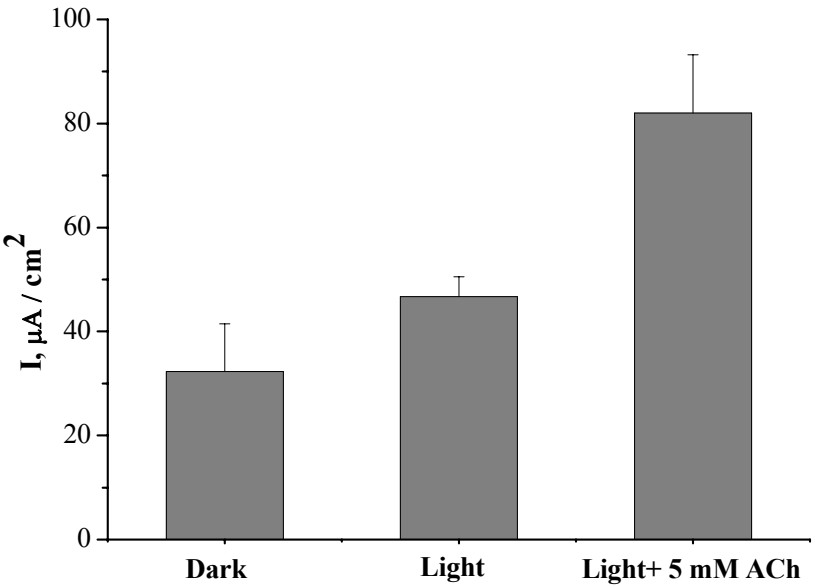

Figure 3.31. The increment of outward current amplitude of *Nitella flexilis* cells after proton pump activation by light and 5 mM ACh application.

Voltage-clamp experiments and voltage current curves revealed the effect of 5mM ACh on H^+–ATPase activation by light. Reversal potential of the light activated *Nitella flexilis* cells shifted in negative direction by 16 ± 3 mV (n=6). Application of 5 mM ACh increased this light caused effect by 15 ± 7 mV (**Figure 3.30).**

It was shown that amplitude of proton pump outward current increased due to the effect of acetylcholine (**Figure 3.31**) Conductance of the light-activated cells after ACh application increased by 1,78 times ($P< 0,01$, n=6).

4. DISCUSSION

4.1. The effect of acetylcholine on Characeaen membrane potentials electrogenesis

Changes in membrane potential after application of ACh indicate effect of ACh on ion transport systems involved in plant potential electrogenesis. The results of this study demonstrate that 5 mM ACh activates K^+ ion channels in the resting state, Cl^- and Ca^{2+} ion channels after excitation and enhances activation of H^+-ATPase in characeaen cells. We have found that ACh elicits depolarization of membrane potential immediately after application. We could propose that ACh increases background conductance. A rise in proton pump activity in 25 min counteracts this increase in background conductance and the resting potential difference remains negative. The same effect of proton pump activation was shown after NaCl impact in Characeaen *Lamprothamnium* (Beilby & Shepherd, 2006b). The effect of ACh on membrane permeability supports this assumption.

Analysis of action potential characteristics in Characean *Nitellopsis obtusa* cells demonstrated that ACh depolarizes membrane potential and prolongs the repolarization. It has been shown that primary mechanism of ACh action in plants was regulation of the membranes permeability to protons (Jaffe, 1970), K^+ (Roshchina, 2001), Cl^- (Gong & Bisson, 2002) and Ca^{2+} (Tretyn A., 1987). It has been established the same ion transport systems to be active in the generation of plant action potentials (Lunevsky, 1983; Trebacz et al., 1996; Plieth & Hansen, 1996; Thiel et al., 1997; Beilby, 2007). Our analysis of *Nitellopsis obtusa* membrane potentials and changes demonstrated in AP

after application of ACh indicate effect of ACh in high concentrations on ion transport systems involved in AP generation. We show that the addition of ACh prolongs the repolarization of the action potential just as Gong and Bisson have demonstrated in another Characeaen *Chara coralina* (Gong & Bisson, 2002). ACh could affect time of repolarization modulating activity of some plasma membrane transport systems. K^+_{out} channels are the main transport system and K^+_{out} current is the main current conditioning process of repolarization. If the K_{out}^+ channels at the plasma membrane were inhibited by ACh we could saw prolongation of AP. Our results clearly showed ACh induced activation of K^+ channels and increase of K^+_{out} and K^+_{in} currents. So we could not attribute reduction of repolarization rate to the inhibition of K^+ channels.

Another transport system which could prolong AP repolarization - H^+-ATPase. Reducing activity of proton pump ACh could reduce flow of positive charges from the cell and prolong repolarization. Our data demonstrated ACh conditioned activation of H^+ ATPase but not inhibition. Furthermore, we noticed major prolongation of repolarisation after inhibition of proton pump by DCCD. Micromolar concentrations of DCCD are sufficient to give complete and irreversible inhibition of proton pumping. K^+ channels proved to be insensitive to DCCD, a classic inhibitor of electrogenic proton pump even at concentration 200 μM (Sokolik & Yurin, 1986). But it was reported that hyperpolarization or depolarization does not necessarily indicate the activity of H^+-pump, and if the H^+-pump activity was reduced, the resting potential could stay at the hyperpolarized level (Tsutsui & Ohkawa, 2001). In this case dynamics of membrane potential after repetitively triggered AP could be a better indicator of proton pump activity. Since the application of DCCD is equivalent to inhibition of the H^+ pump current (Kishimoto, 1984), and our results show that effect of ACh on repolarisation is not dependent on inhibition of proton pump we cannot rule out the possibility that inhibition of H^+-ATPase disturbed homeostasis of cell ions and cell became more sensitive to ACh. However, this inhibition allows a closer look at the different phases of the action potentials. For example, effect of ACh on background conductance and increased depolarizion could be counterbalanced by the activity of H^+-ATPase.

Another possibility is that K^+ and Cl^- inflow could be caused by the gradient and negative voltage generated by proton pump. We found that ACh activates K^+_{in} channels. If Cl^- enters the cell by $2H^+/Cl^-$ cotransport system, DCCD indicates effect of ACh on

Cl^- efflux. Therefore ACh increases duration of repolarization by modulating activity of Cl^- channels. Using voltage clamp method we proved effect of ACh on Cl^- conductance. We found threefold increase of Cl^- current in 5 mM ACh solution. ACh determined increased efflux of negative charges during AP generation, which could not be compensated adequately by activity of K^+ channels and H^+ -ATPase. It can explain prolongation of the fast repolarization as well as the slow repolarization.

The effect of ACh on Cl^- conductance could be mediated by activating the same channels which are involved in AP generation and keeping them longer in active state or by activating channels of another type (for example NSCCs channels) which become permeable for Cl^-. Furthermore, ACh could increase permeability of Cl^- channels in plasmalemma and tonoplast. Patch clamp investigation of Cl^- channels in *Chara coralina* tonoplast demonstrated increased probability of open state which would keep cell more depolarized (Gong & Bisson, 2002). It was found that increase in Cl^- concentration in the cytoplasm causes increase in Cl^- current (Beilby & Shepherd, 2006b). To activate tonoplast Cl^- channel of *Characeae* ACh must cross the plasma membrane and enter the cytoplasm. The effect of ACh in our experiments was reversible thus either ACh could not enter cytoplasm (then activation of Cl^- channels is possible by the action of second messengers (like Ca^{2+}) or ACh was hydrolyzed.

We have demonstrated an enhanced excitability of Nitellopsis obtusa cells after ACh application as cells started to generate AP at more negative voltages. Plant APs is caused by activity of Ca^{2+}-dependent Cl^- channels in the plasma membrane and the tonoplast (Lunevsky et al., 1983). It was shown Ca^{2+}-dependent Cl^- channels opened most frequently between approximately –80 and –100 mV (Okihara K. et al., 1991). Our results in APW solution have confirmed these data. It is possible, that ACh have facilitated opening of Ca^{2+}-dependent Cl^- channels at more negative voltages and reduced excitation threshold after alteration in physical characteristics of plasma membrane. ACh caused increase in excitability could be explained by increased permeability to Ca^{2+}. It has been shown that ACh had increased intracellular Ca^{2+} concentration (Tretyn,. 1987). It is possible that ACh accelerates accumulation of Ca_{cyt}^{2+} required for Ca^{2+}-dependent Cl^- channels activation or there are acetylcholine receptors permeable to Cl^- in the cell membrane or inside the cell (Gong & Bisson, 2002). We found increased permeability to Ca^{2+} after ACh application and resulting rise of cytosolic Ca^{2+} concentration could cause

reduction of Cl^- current latency. ACh mediated prolongation of Cl^- current inactivation could be determined by increasing time of the Cl^- channels in open state. In this case acetylcholine could have inhibitory effect and could slow down rate of inactivation of Cl^- channels participating in excitation (Gong & Bisson, 2002). Otherwise, keeping longer increased intracellular Ca^{2+} concentration could prolong time Cl^- channels are in open state (Beilby & Shepherd 2006b). The cytoplasmic concentration of Ca^{2+} is maintained by two transport systems. It is proposed that physiological role of Ca^{2+}/H^+ antiporters is to remove large amount of Ca^{2+} from cytosol after Ca^{2+} signal, while Ca^{2+}-ATPases maintain very low level of Ca^{2+} during resting state (Hirschi, 2001). Consequently prolongation of AP duration we found after DCCD application could be explained by longer time Cl^- channels are in open state after increase of intracellular Ca^{2+} concentration.

4.2.Acetylcholinesterase activity in Characeaen cells.

Enzymatic cleavage of ACh molecule during the ACh hydrolysis is performed via activity of acetylcholinesterase. Based on data from our electrophysiological expriments we supposed AChE activity in Characeaen cells. It is agreed that AChE is widely distributed in the plant kingdom (Sagane et al., 2005; Momonoki, 1997). 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 as are controlled by endogenous ACh. Indirect possibility of AChE activity was shown in Characeae by Dettbarn (Dettbarn, 1962) who found that *Nitella* is capable of hydrolyzing acetylcholine up to 6,5 pmoles/g/hr. From our experimental data we can make an assumption that inhibitors of AChE enhancing amount of ACh in the apoplast facilitate ACh binding to the membrane or its accessibility to the cytoplasm. In many cases plant AChE activity was found in cell wall, but it was established in apoplast and inside the cell as well (Fluck & JaVe, 1974c; Fletcher et al., 2004). Effect of high exogenous ACh can be accounted for by its poor penetration into the plants' tissues and/or its rapid hydrolysis too. The high dose of ACh released inside the cell would have been expected to overcome a possible cholinesterase activity in the cell wall. We found maximum effect on AP repolarization in *Nitellopsis obtusa* cells at 5 mM ACh. This fits well to the data presented by Gong and Bison (Gong

& Bisson, 2002) where they found maximum effect of ACh on *Chara coralina* cells membrane potential at concentrations 1 to10 mM. It was shown that AChE activity of various plants was stimulated when ACh concentration was less than 0,5 mM, and inhibition of AChE was observed when ACh concentration was within range 1-6 mM, (Wišniewska & Tretyn, 2003). We also observed that prolongation of ACh application time enhanced depolarizing effect.

The enzymatic activity of AChE has been shown to be altered by the environmental contaminants such as metals. It was shown that Cd^{2+} significantly inhibited AChE activity *in vitro* (Frasco et al., 2005). We performed *in vivo* experiment aimed to investigate effect of Cd^{2+} on membrane potentials in *Nitellopsis obtusa* cells. However cadmium is a heavy metal which is classified as a toxicant in plants. Addition of 0,1 or 1 mM Cd^{2+} to the experimental solution caused strong depolarization. Cd^{2+} toxic effects on membrane permeability can be attributed to the influence on the transport systems, involved in plant membrane electrogenesis (Karcz &Kurtyka, 2007; Astolfi et al., 2005; Moran et al., 1990). Interaction between calcium signalling and cadmium in plant cells has been recently demonstrated in Arabidopsis suspension cells (Perfus-Barbeoch, 2002). Decrement of AP peak found in our experiments after Cd^{2+} treatment could be explained by impact of Cd^{2+} on the intracellular calcium level. However it is supposed existence of some mechanism in the plant systems able to resist toxic effect of Cd^{2+} up to certain concentrations (Cobbett & Goldsbrough 2002). We found that threshold Cd^{2+} concentration for *Nitellopsis obtusa* cell is 20 μM when membrane potential is negative more than -220 mV. We assume that in our short lasting experiments (few hours) Cd^{2+} at 20 μM concentration acts more as acetylcholynesterase inhibitor than affecting membrane transport systems at membrane potential 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. After application of specific AChE inhibitor neostigmine bromide, which itself has no influence on membrane potential dynamics, we have found the same effect on the amplitude of the second AP. We have shown that Cd^{2+} and NB

strengthened the depolarizing effect of acetylcholine on membrane potential repolarisation after the first stimulation.

4.3. Cholinergic system and signalling patways in plants

We have found that application of micromolar concentrations of nicotine caused depolarization of MP, effect on the second AP and generation of double action potentials. It could be concluded that the comparable effect could be elicited by action of nicotine in low concentrations and ACh in high concentration. Patch clamp experiments have showed synergistic effect of 4 mM ACh and 6 mM nicotine synergistic effect on Cl^- channels opening probability in *Chara* cells (Gong & Bisson, 2002), but influence of nicotine on membrane potentials was not investigated in this work. Our data on efficacy of nicotine supposed existence of AChE in Characeaen cells. Moreover, these results are in consistence with notion about existence of ACh binding sites in characeaen membranes and allows assumption - if ACh receptors exist in *Nitellopsis obtusa* cells, they are nicotinic. ACh receptors were shown in *Vicia faba* stomata cells and they were defined as nicotinic (Wang, 1998) and muscarinic ACh receptors (Meng et al., 2004). Acetylcholine channels found in wheat leaf cells were called nicotinic ACh receptors (Madhavan and Pinkerton, 1997). Depolarization and spontaneous activity of the cell observed after nicotine application was reversible by contrast to application of Cd^{2+}. Therefore we could conclude that the effect of ACh and nicotine was not toxic to the *Nitellopsis obtusa* cells.

We propose that amplitude and duration of AP and membrane potential dynamics triggered by repetitive stimulation has not only the physiological implications but could be used in ecotoxicology. Repetitive action potentials could not only reflect the impact of various compounds, but they could accelerate death of the cells (Shepherd, 2008). Moreover. We found that if Characeaen cells membrane potential reached more negative values, cells became more resistant to toxic effect of various compounds.

It has been proposed (Shabala et al., 2006) that frequency modulation, not only amplitude modulation, may be used for encoding of environmental information in plants. It is possible that keeping the cell in more depolarized state after the first AP in ACh solution has the physiological implications. Probably the second AP causes accumulation

of Ca^{2+} sufficient to exert physiological effect. 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*, (Trebacz et al., 1996, Volkov et al., 2008) but in other plant species too. Assuming the second AP has notional physiological significance, we could propose participation of ACh in electrical signal transmission. Ion fluxes, amplitude, duration and dynamics of repetitively evoked electrical signals could play a key role in the generation of the physiological response in plant.

5. CONCLUSIONS

1. Acetylcholine activates main membrane transport systems involved in electrogenesis of Characeaen membrane potential:
    a. K^+ channels at the resting state and photoinduction in *Nitella flexilis* cells
    b. Cl^- and Ca^{2+} channels after excitation in *Nitellopsis obtusa* cells

2. Acetylcholine and nicotine prolong repolarization of action potentials.

3. The inhibitors of acetylcholinesterase - dimetoat, Cd^{2+} and neostigmine bromide - strengthened the effect of acetylcholine on electrophysiological characteristics of Characeaen membrane.

4. Acetylcholine and nicotine increase the excitability of *Nitellopsis obtusa* cells.

5. Spontaneous activity in *Nitellopsis obtusa* cells could be evoked by membrane potential depolarization exceeding excitation threshold level.

SANTRAUKA

Vilma Kisnierienė

ACETILCHOLINO ĮTAKA MENTURDUMBLIŲ LĄSTELIŲ MEMBRANŲ PERNAŠOS SISTEMOMS

Pagrindinis disertacijos tikslas buvo nustatyti acetilcholino (ACh) poveikį menturdumblių plazminės membranos pernašos sistemų funkcionavimui ir veikimo potencialų generavimo dinamikai. Darbas buvo atliekamas naudojant standartinę mikroelektrodinę techniką, standartines biopotencialų matavimų procedūras ir fiksuotos įtampos metodą. Pirmą kartą, įvertinant cheminių medžiagų poveikį menturdumblių ląstelių membraninių procesų funkcionavimui, buvo panaudotos veikimo potencialų serijos. Darbo metu buvo tiriama, kaip ACh veikia menturdumblių (*Nitella flexilis* ir *Nitellopsis obtusa*) bendruosius elektrinius parametrus ir atskiras jonų, dalyvaujančių menturdumblių elektrogenezėje, pernašos sistemas. Buvo tiriamas acetilcholinesterazės inhibitorių bei suminis šių inhibitorių ir ACh poveikis menturdumblių ląstelių elektrofiziologiniams parametrams. Taip pat ištirtas nikotino, kuris yra ACh agonistas, poveikis menturdumblių ląstelių elektrofiziologiniams atsakams. Nustatyta, kad dėl 5 mM ACh poveikio aktyvuojami K^+ kanalai ramybės būsenoje, Cl^- bei Ca^{2+} kanalai sužadinimo metu bei sustiprinama H^+-ATPazės aktyvacija. Depoliarizuojančiu poveikiu menturdumblių ląstelių membranoms pasižymi tiek ACh, tiek nikotinas. Nustatyta, kad ACh didina menturdumblių *Nitellopsis obtusa* ląstelių jaudrumą. Parodyta, kad elektrofiziologiniais tyrimo metodais galima parodyti acetilcholinesterazės veikimą menturdumblių ląstelėse. Menturdumblių membraninio potencialo dinamikos analizė veikimo potencialų serijos metu gali būti naudojama vykdant ekologinę aplinkos užterštumo stebėseną.

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