Physiological and Hemolytic Toxicity of Some Aminophosphonates

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The effect of novel synthesized aminophosphonates on membrane potential and electrical conductance of internodal cells of *Nitellopsis obtusa* and hemolysis of erythrocytes (RBC) was studied. It was found that those the organophosphorous compounds, when present at 10–100 μM concentrations, caused depolarization and increased electrical conductance of alga membranes. They also influenced fluidity of erythrocyte membranes. When used at higher concentrations aminophosphonates caused hemolysis of RBC. The changes observed depended on structural features of the aminophosphonates, i.e., substituents at the carbon, phosphorus and nitrogen atoms, and, most probably, may be the result of direct interaction of the aminophosphonates with the lipid phase of the plasma membrane and the induced structural changes. Two modes of interaction are proposed.

Introduction

The aminophosphonates belong to an interesting class of plant growth regulators, first synthesized over 50 years ago (Pikl 1943). Since that time many organophosphorous compounds have been found to be potent pesticides (Guenther and Loettge, 1971; Baird *et al.*, 1972; Perkow, 1983/1988; Czerwiński *et al.*, 1982; Gancarz *et al.*, 1985; Gancarz and Dudek, 1986; Kochmann *et al.*, 1986; Forlani *et al.*, 1987). The biological activity of some of those compounds was suggested to be correlated with their hydrophobicity (Gancarz and Dudek, 1986), althought this activity may, in some cases, be a consequence of direct involvement of organophosphorous compounds in metabolic processes (Linsel *et al.*, 1988). Generally, biological activity of organophosphorous compounds relates to their interaction with the lipid or protein phases of biological membranes (O'Brien, 1979; Crowley, 1980; Linsel *et al.*, 1988; Di Tomaso, 1993; Shimakuburo, 1994; Sterling, 1994; Marré *et al.*, 1998). In this paper we present the results of studies on electrophysiological and hemolytic properties of some newly synthesized aminophosphonates expected to correlate with their biological activity. The role of different structural parts of aminophosphonates in that activity is discussed.

Materials and Methods

Fresh internodal cells *of Nitellopsis obtusa* were used. The alga was taken from the natural environment in September – October period (Zagłębocze Lake close to Sosnowica, Lublin region, Poland) and kept in a laboratory aquarium for several months under natural illumination.

Single internodal cells (mean dimension: 0.4 mm diameter and 3.1 cm length) were incubated for 24 h in darkness in measurements solution (artificial pond water – APW).

The control solution of pH 7.0 contained 1 mM NaCl, 0.1 mM KCl and 0.1 mM $CaCl_2$. The compounds studied were synthesized in the Institute of Organic Chemistry, Biochemistry and Biotechnology, Technical University of Wrocław. Synthesis details as well as spectral data were given earlier (Gancarz *et al.*, 2000; 2001). The chemical structures of the investigated compounds and substituents are shown in Table I. The incubation solutions containing these compounds were prepared by dissolving the compound in a small amount of ethanol (1 ml/1 l of solution) and then in APW. The same amount of ethanol was added to the control APW solution.

Membrane potential and electric conductance were measured in a routine way (Fig. 1). The potential difference between vacuole and external medium was measured with one pair of microelec-

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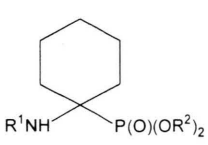
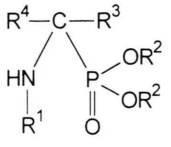
Table I. General structure and substituents of the compounds studied.

Comp.	R^4	R^3	R^2	R^1
1	CH_3	n-C_3H_7	n-C_4H_9	n-C_4H_9
2	CH_3	n-C_4H_9	n-C_4H_9	n-C_5H_{11}
3	-C_5H_{10}		C_2H_5	n-C_8H_{17}
4	-C_5H_{10}		n-C_4H_9	n-C_8H_17
5	-C_5H_{10}		n-C_4H_9	C_2H_4OH





trodes filled with 3 M KCl. In the current circuit Ag/AgCl electrodes were used.

The cells were placed in a three-compartment container. The compartments were filled with a liquid and partitioned by narrow empty spaces that insured electric isolation between the compartments. The middle compartment was perfused with control solution or solution containing a modifier at a rate of 1 ml/min. In that part of the container current and membrane potential were measured. The middle compartment contained 3 mm of the alga cell.

The electric conductance of the modified membrane fragment was determined by the voltage response to a constant signal of ± ca. 20 nA of 200 ms duration and 10 s repetition time.

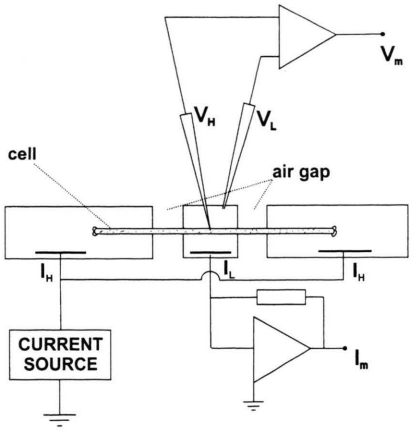


Fig. 1. Block diagram of measurement setup. V_H, V_L – voltage microelectrodes, I_H, I_L – current Ag/AgCl electrodes, V_m, I_m - analog signals of membrane voltage and current. Volume of central of chamber where V_m and I_m were measured was 0.5 ml. Potential measuring microelectrode (V_H) tip diameter was 1 μm. Reference electrode (V_L) tip diameter was 10 μm.

All experiments were conducted in darkness at room temperature (22 ± 1 °C).

Fresh heparinized pig blood was used in the hemolytic experiments. Erythrocytes (RBC) were washed four times in phosphate buffer of pH 7.4 and incubated in it, after adding chosen concentrations of aminophosphonates (0.5, 1.0, 1.5, 2.0 and 2.5 mM), at 37 °C for 4 h. The hematocrit (percent of erythrocytes in suspension) was 2%. Percent of hemolysis was measured of 1 ml samples taken after 0.5, 1, 1.5, 2, 3 and 4 h of incubation. They were centrifuged and the hemoglobin content in the supernatant was measured with a Specol 11 spectrophotometer (Carl Zeiss, Jena, Germany) at 540 nm.

Plotted kinetic curves of hemolysis were then used for calculation of the concentration of compound inducing 50% (C_{50}) and 100% hemolysis (C_{100}). Percent values of hemolysis after 1.5 h modification were used.

Fluidity experiments were done on erythrocyte ghosts using the compounds studied in 0.01 mM and 0.02 mM concentrations. The fluorescent probe used was TMAP-DPH at 1 μM concentration. The measurements were performed with SFM 25 spectrofluorometer (KONTRON, Zurich, Switzerland) at 25 °C. The excitation and emission wavelengths were 354 nm and 429 nm, respectively. The anisotropy coefficient A was calculated according to the formula (Lakowicz, 1983; Campbell and Dwek, 1984; Lentz, 1988):

$$A = (I_{II} - GI_{\perp})/(I_{II} + 2GI_{\perp}) \quad (1)$$

where I_{II} – intensity of fluorescence emitted in direction parallel to the polarization plane of the exciting light, $I_{\perp}$ - intensity of fluorescence emitted in the perpendicular direction and G – diffraction constant.

All reagents used were of analytical grade. The fluorescent probe TMAP-DPH {[1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene]-p-toluenesulfonate} was purchased from Molecular Probes Inc. (Eugene, Oregon, USA).

Results

All compounds investigated generally depolarized the plasma membrane of *Nitellopsis obtusa* and increased the membrane conductance. The ef-

fect depended on the chemical structure of the modifier and its concentration. Typical results obtained are presented in Figs. 2–3, where compound **3** was chosen as an example. The results obtained for other aminophosphonates were qualitatively the same. As shown in Fig. 2A, the resting potential slowly decreased after addition of 20 μM compound **3** to the bathing solution. The membrane conductance (Fig. 3) increased, but a transient decrease was also observed. The values of the resting potential as well as conductances reach steady-state values after approx. 15 min incubation in the modifier-containing solution. We have not observed changes in the membrane resting potential of membrane and conductance at a modifier concentration below 20 μM. The depolarization and the conductance increased with an increase in modifier concentration. 100 μM caused irreversible destruction of the plasma membrane. The modifier-induced strong depolarization triggered an action potential (quasi a.p.), with a characteristic slow repolarization phase which ended below the value of the resting potential (Fig. 2A).

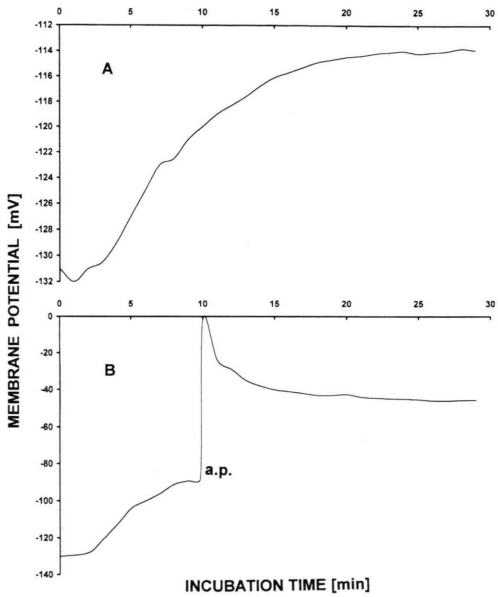


Fig. 2. A. Time course of potential difference between vacuole and external medium (RP) in the presence of 20 μM of compound **3**. B. Time course of potential difference between vacuole and external medium (RP) in the presence of 100 μM of compound **3** (a.p. – action potential).

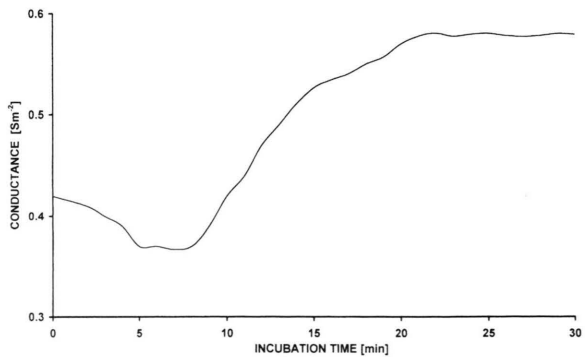


Fig. 3. Time course of membrane specific conductance in the presence of 20 μM of compound **3**.

Discussion

The efficacy of the aminophosphates to cause depolarization follows the sequence (see Table II): **4** < **1** < **3** < **5** < **2**. The strongest modifying property exhibited the acyclic compound **2**. The second acyclic compound was not so effectively influencing the membrane potential, probably due to its smaller lipophilicity. The best modifier of the cyclic aminophosphonates was compound **5**. Its efficacy did not seem to be the result of its lipophilicity, as it has shortest alkyl chain substituted at the nitrogen atom with a terminal hydroxyl group. Also, the very weak efficacy of compound **4** did not correspond to its high lipophilicity as compared with compound **3** (see Table II). High lipophilicity of biologically active compounds was shown previously to be a very important factor influencing the electrophysiology of the plasma membrane of *Nitellopsis obtusa* (Trela *et al.*, 1990; Przestalski *et al.*, 1991). Hemolysis of erythrocytes is generally assumed to be the result of the action of exogenic agents on the lipid phase of erythrocytes (Kleszczyńska *et al.*, 2000), and any agreement between hemolysis and electrophysiological experiments should point at the lipophilicity of aminophosphonates studied as the most important factor deciding their biological activity. The results of hemolytic experiments (see Table II) gave the following sequence of hemolytic efficiency of aminophosphonates: **4** < **5** < **1** < **3** < **2**. Excluding compound **5**, this sequence is identical as found for electrophysiological efficacy of the aminophosphonates. Thus, it seems that in the case of compounds **1**, **2**, **3** and **4** their lipophilicity and the disturbance of the lipid phase of the alga mem-

Table II. Effect of 20 μM of aminophosphonates on the resting potential in cells of *Nitellopsis obtusa*, concentration of the aminophosphonates causing 50% (C_{50}) and 100% hemolysis of erythrocytes (C_{100}), and the value of anisotropy coefficients $A_{0.01}$ and $A_{0.02}$ for 0.01 mM and 0.02 mM concentration of aminophosphonates.

Comp.	Control value of the resting potential [mV]	Resting potential 15 min. after addition of the modifier [mV]	Relative changes of the resting potential %	C_{50} [mM]	C_{100} [mM]	$A_{0.01}$	$A_{0.02}$
1	−134 ± 6.4 (3)	−112 ± 6.8	16.4	0.24	0.30	0.247	0.232
2	−128 ± 7.1 (3)	−92 ± 9.1	28.1	0.17	0.25	0.250	0.235
3	−131 ± 5.7 (3)	−106 ± 7.1	19.1	0.20	0.25	0.256	0.242
4	−129 ± 3.3 (5)	−118 ± 8.1	8.5	1.50	2.50	0.261	0.255
5	−133 ± 6.2 (3)	−98 ± 7.9	26.3	0.35	0.60	0.250	0.248
Control	–	–	–	–	–	0.275	0.275

Data for electrophysiological experiments are ± SE of the number of experiments indicated in parentheses. Standard deviation for hemolytic experiments did not exceed 4%.

brane are deciding factors for the observed changes in membrane potential and conductance. Since it was shown (Berglund *et al.*, 2000; Kasamo *et al.*, 2000) that besides other environmental factors, this kind of interaction should be accompanied by fluidity changes in the lipid phase, we have also measured the anisotropy of fluorescent probes incorporated into erythrocyte membranes. The measured values of the anisotropy coefficients ($A_{0.01}$ and $A_{0.02}$) did not differ very much for all aminophosphonates studied (Table II). As expected, the most extensive fluidity changes were found for acyclic compounds. Once again compound **4** exhibited the weaker modifying properties. Also, compound **5**, like in hemolytic experiments, was weaker fluidizing erythrocyte membranes. It seems that this compound, in contrast to all other aminophosphonates, may rather influence significantly the potential barrier at the surface of the lipid bilayer or directly interact with the protein part of the plasma membrane.

Acknowledgments

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