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Influence of decavanadate on rat synaptic plasma membrane ATPases activity

Danijela Krstić[1], Mirjana Čolović[2], Nada Bošnjaković-Pavlović[3], Anne Spasojević-De Bire[4] and VesnaVasić[2]

[1] *Institute of Medicinal Chemistry, University School of Medicine, Belgrade, Serbia*
[2] *Department of Physical Chemistry, Vinča Institute of Nuclear Sciences, Belgrade, Serbia*
[3] *Faculty of Physical Chemistry, University of Belgrade, Serbia*
[4] *Ecole Centrale Paris, Laboratoire SPMS UMR CNRS 85801, Grande Voie des Vignes, 92295 Châtenay-Malabry, France*

Abstract. The *in vitro* influence of decameric vanadate species on Na^+/K^+-ATPase, plasma membrane Ca^{2+}-ATPase (PMCA)-calcium pump and ecto-ATPase activity, using rat synaptic plasma membrane (SPM) as model system was investigated, whereas the commercial porcine cerebral cortex Na^+/K^+-ATPase served as a reference. The thermal behaviour of the synthesized decavanadate (V_{10}) has been studied by differential scanning calorimetry and thermogravimetric analysis, while the type of polyvanadate anion was identified using the IR spectroscopy. The concentration-dependent responses to V_{10} of all enzymes were obtained. The half-maximum inhibitory concentration (IC_{50}) of the enzyme activity was achieved at $(4.74 \pm 1.15) \times 10^{-7}$ mol/l for SPM Na^+/K^+-ATPase, $(1.30 \pm 0.10) \times 10^{-6}$ mol/l for commercial Na^+/K^+-ATPase and $(3.13 \pm 1.70) \times 10^{-8}$ mol/l for Ca^{2+}-ATPase, while ecto-ATPase is significantly less sensitive toward V_{10} ($IC_{50} = (1.05 \pm 0.10) \times 10^{-4}$ mol/l) than investigated P-type ATPases. Kinetic analysis showed that V_{10} inhibited Na^+/K^+-ATPase by reducing the maximum enzymatic velocity and apparent affinity for ATP (increasing K_m value), implying a mixed mode of interaction between V_{10} and P-type ATPases.

Key words: Decavanadate — Na^+/K^+-ATPase — Plasma membrane Ca^{2+}-ATPase — ecto-ATPase

Introduction

Interest in the interaction of vanadate oxoanions with biological systems has increased since it has been demonstrated to have a variety of physiological effects acting either as a phosphate analogue in the monomeric form ($H_2VO_4^-$) (Cantley et al. 1977) or through oligomeric (dimeric, tetrameric and decameric) vanadate species which interact with many biomolecules with various and versatile activity (enzymes inhibitor or activator). Recent data indicate spermicidal and anti-HIV activity of vanadium compounds (D'Cruz et al. 2003) and the use of these compounds for the treatment of diabetes (Heinemann et al. 2003; Sakurai et al. 2004; Yang et al. 2004) as well as the prevention of animal carcinogens (Kanna et al. 2004; Molinuevo et al. 2004). It was reported that decavanadate (V_{10}) is the major protein-bound species of vanadium and have a stronger effect on various enzymes, when compared to other vanadate oligomers (Aureliano and Gândara 2005). Recently reported V_{10} studies include the possibility of its use as a tool in the understanding of the transducing chemical energy into conformational energy for Ca^{2+} transport by Ca^{2+}-ATPase (Stokes et al. 2005) as well as molecular mechanism of muscle contraction (Tiago et al. 2004). Inhibitions of several adenosine triphosphatases (ATPases) such as P-type ATPases, ABC-ATPases and ribonucleases by V_{10} suggest that V_{10} interactions with these proteins probably favored by the existence of an ATP binding site (Messmore and Raines 2000; Pezza et al. 2002).

Na^+/K^+-ATPase (sodium pump) and plasma membrane Ca^{2+}-ATPase (PMCA)-calcium pump belong to the P-type ATPase family, the members of which are able to utilize the energy of ATP to transport ions against their electrochemical gradient across membrane and strongly inhibited by

Correspondence to: Danijela Krstić, Institute of Medicinal Chemistry, University School of Medicine, Višegradska 26, 11 000 Belgrade, Serbia
E-mail: krsticdana@yahoo.com

nanomolar concentration of orthovanadate (Cantley et al. 1977).

Na^+/K^+-ATPase is a cell membrane located enzyme that establishes and maintains the high internal K^+ and low internal Na^+ concentrations, characteristic and essential for normal cellular activities of most animal cells (Vasilets and Schwarz 1993; Rodriguez de Lores Arnaiz and Pena 1995). The activity of this enzyme is very sensitive to the presence of some metal ions and organic compounds of various structures, especially some drugs and pesticides (Blasiak 1995; Krstić et al. 2004, 2005). PMCA is a fine tuner of cytosolic calcium concentration in excitable cells, while the calcium pump is the sole system responsible for extrusion of calcium ions outside nonexcitable cells (Zylinska and Soszynski 2000). The ecto-ATPase, which does not belong to the P-type ATPase family, represents an integral membrane protein that, in the presence of divalent cations (Ca^{2+} or Mg^{2+}), hydrolyses extracellular nucleotides because of the outward orientation of its active site (Nagy 1986). By hydrolysing ATP to ADP, ecto-ATPase represents the major inactivating agent in purine-triphosphate signalling (Horvat et al. 2006). The aim of this work was the investigation of the *in vitro* effect of ammonium V_{10}, $(NH_4)_6V_{10}O_{28}{\cdot}5H_2O$ on Na^+/K^+-ATPase (sodium pump), PMCA-calcium pump and ecto-ATPase (Mg^{2+}-ATPase) activity, using rat synaptic plasma membrane (SPM) as a model system, while the commercial porcine cerebral cortex Na^+/K^+-ATPase served as a reference. In addition, extensive kinetic studies of porcine cerebral cortex Na^+/K^+-ATPase were undertaken to determine the nature of enzyme inhibition by V_{10}. These enzymes were chosen because of their key role in normal functioning most cells of higher eukaryotic organisms (Scheiner-Bobis 2002) as well as pivotal roles in cancer cell migration (Lefranc et al. 2008) and, on the other hand, known influence of V_{10} on nucleotide-dependent enzymes (Boyd et al. 1985; Csermely et al. 1985; Varga et al. 1985; Aureliano 2000; Pezza et al. 2002; Aureliano and Gândara 2005).

Materials and Methods

Chemicals

All chemicals were of analytical grade. Na^+/K^+-ATPase from porcine cerebral cortex and ATP were purchased from Sigma Co., as well as some chemicals for medium assay (magnesium chloride and Tris-HCl). The specific Na^+/K^+-ATPase activity was 25.8 μmol Pi/h/mg protein (Pi – inorganic orthophosphate). Other medium assay chemicals (sodium chloride, potassium chloride), chemicals for determination of Pi (stannous chloride and ammonium molybdate) and ammonium trioxovanadate (NH_4VO_3) used for synthesis were from Merck (Darmstadt, Germany).

Synthesis of ammonium V_{10}

The title compound, $(NH_4)_6V_{10}O_{28}{\cdot}5H_2O$, was prepared by dissolving NH_4VO_3 (0.4 g, 3.42 mmol) in distilled water (20 ml) as described in Chinea et al. (2000) to obtain V_{10} anions. The solution was stirred and heated until complete dissolution of NH_4VO_3 (about 2 h). The pH was adjusted to 5.90 by dropwise addition of NH_4OH. Orange crystals were formed within four days from the solution, kept at room temperature.

Thermal studies

Thermal analysis was performed on Instruments model SDT 2960 Simultaneous DSC-TGA (differential scanning calorimetry-thermogravimetric analysis) under following conditions: air atmosphere, sample mass 100 mg, heating rate $10°C{\cdot}min^{-1}$, temperature interval 20–700°C.

Infrared (IR) spectroscopy

The IR spectra in pastille was recording on Thermo Scientific spectrometer Nicolet 6700FT-IC, at room temperature, in the range 450–4000 cm^{-1}. The solid compound was mixed with potassium bromide into transparent disk.

Preparation of V_{10} solution

Ammonium V_{10} stock solution (10 mmol/l) was prepared by diluting 117.3 mg of V_{10} in 10 ml of water and held in refrigerator until use. Working solutions were prepared daily by diluting stock solution to desired concentrations.

SPM preparation

SPM were isolated from the whole brain of 3-month-old male Wistar albino rats from the local colony. Animals were kept under controlled illumination (lights on: 5:00 a.m.–5:00 p.m.) and temperature (23 ± 2°C), and had free access to food and water. The Guiding Principles for the Care and Use of Animals based upon Helsinki Declaration (1964) and Protocol of the "Vinča" Institute on Care and Treatment of Laboratory Animals were strictly followed. After decapitation with a guillotine (Harvard Apparatus), brains were rapidly excised and pooled (6/pool) for immediate preparation of SPM isolated according to the method of Cohen et al. (1977), as modified by Towle and Sze (1983). The mitochondrial contamination and protein content were determined according to the standard procedure (Nikezić et al. 1998.) SPM were stored at –70°C until used.

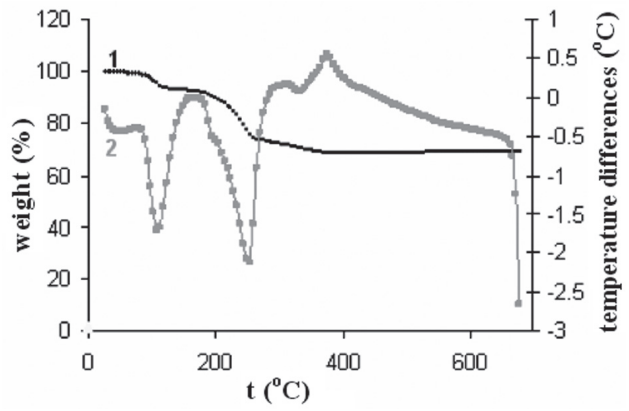

Figure 1. Thermogravimetric analysis (line 1) and differential scanning calorimetry (line 2) curves for the thermal decomposition of compound $(NH_4)_6V_{10}O_{28}{\cdot}5H_2O$.

ATPase assays

The standard assay medium for investigation of Na^+/K^+-ATPase activity contained (in mmol/l): 50 Tris-HCl (pH 7.4), 100 NaCl, 20 KCl, 5 $MgCl_2$, 2 ATP and 125 mg/l SPM proteins (i.e. 290 mg/l commercial porcine cerebral cortex proteins) while the rat synaptic PMCA activity was assayed in standard medium containing (in mmol/l): 50 Tris-HCl (pH 7.4), 0.5 EGTA, 0.5 $CaCl_2$, 5 $MgCl_2$, 2 ATP and 125 mg/l SPM proteins in a final volume of 200 µl. Assay for SPM ecto-ATPase activity contained (in mmol/l): 50 Tris-HCl (pH 7.4), 5 $MgCl_2$, 2 ATP and 125 mg/l SPM proteins. After preincubation for 10 min at 37°C in the absence (control) or in the presence of investigated compound, the reaction was initiated by addition of ATP and stopped after 10 min by adding 22 µl ice cold of 3 mol/l $HClO_4$ and immediate cooling on ice. The released Pi liberated from the hydrolysis of ATP was determined by a modified spectrophotometric method (Vasić et al. 1999). The spectrophotometric measurements were performed on a Perkin Elmer Lambda 35 UV VIS spectrophotometer. The activity obtained in the presence of Mg^{2+} alone was attributed to ecto-ATPase activity. SPM Na^+/K^+-ATPase as well as SPM Ca^{2+}-ATPase activities were calculated by subtracting the Mg^{2+}-ATPase (ecto-ATPase) activity from the total ATPase activity in the presence of Na^+, K^+ and Mg^{2+} ions (i.e. in the presence of Ca^{2+} and Mg^{2+} ions). The results are expressed as mean percentage enzyme activity compared to the corresponding control value ± S.E.M. of at least three independent experiments done in triplicate.

Kinetic analysis

Kinetic experiments were carried out according to the slightly modified method of Philips (Philips et al. 1978) using the commercial porcine cerebral cortex Na^+/K^+-ATPase. The initial velocities were measured in the same incubation medium as a function of rising concentrations of $MgATP^{2-}$ (0.1–5.0 mmol/l). The measurements were performed in the absence and presence of V_{10}, while maintaining the concentrations of other ions (Na^+, K^+ and Mg^{2+}) constant. The experimental data were fitted to the Michaelis-Menten equation by nonlinear regression analysis using EZ-FIT program (Perrella 1988). Maximum enzymatic velocity (V_{max}) and Michaelis constant (K_m) values were derived by nonlinear regression fitting (hyperbola function) of the experimental data as well as from a Lineweaver-Burk plot and expressed in µmol Pi/h/mg protein and mmol/l of ATP. Results are given as means ± S.E.M.

Results

Thermal studies

Thermal decomposition of ammonium V_{10} starts at 85°C, and proceeds as a more step process (Fig. 1). All endo- and exothermic effects are connected with weight changes. The first endothermic step corresponds to the loss of the crystallization water molecules (85–155°C). This crystal form is stable up to 175°C. This step corresponds to the weight loss about 8% and to the departure of about five water molecules *per* V_{10}. After this temperature, there are an endothermic step (180–300°C) and another exothermic step (330–400°C). They correspond to exothermic processes arising from the combustion of organic groups. At about 400°C, the thermostable form, V_2O_5, was formed. Thermogravimetric analysis curves show that the synthesized V_{10} contains 5 molecules of water at room temperature.

IR spectra

The IR spectroscopy is a good method of identification of the type of polyvanadate anion as the structures of these anions are relatively rigid. They are only slightly influenced by the nature of cation and by hydrogen bonds between cation and anion or between crystal water and anion, respectively. Therefore, the synthesized compound could be identified based on their IR spectra (Fig. 2).

The IR spectra of the synthesized V_{10} contains the bands which are characteristic for the groups: NH_4^+, OH^- and V-O. The characteristic bands for NH_4^+ is at 1400 cm^{-1}, bending, and for OH^- at about 1607 cm^{-1}. In the 450–1000 cm^{-1} region the IR spectra of the V_{10} exhibits bands corresponding to V-Ot stretching at 950 cm^{-1} and to V-Ob stretching at 809 cm^{-1}, all of them with position characteristic for V_{10} (Escobar and Baran 1981).

The in vitro influence of V_{10} on SPM ATPase activity

The influence of V_{10} on SPM ATPases and commercial porcine cerebral cortex Na^+/K^+-ATPase activity was investigated by *in vitro* exposure to the enzymes in the concentration range from 1×10^{-10} to 1×10^{-3} mol/l. The results show that increasing concentrations of V_{10} induce inhibition of enzymatic activity in a concentration-dependent manner in all cases (Fig. 3a). The dependence of enzyme activity, expressed as a percentage of the control value (obtained without inhibitor), on inhibitor concentration fits a sigmoidal function (Eq. (1)) for all enzymes. The half-maximum inhibitory concentrations (IC_{50}) of the investigated compound for all ATPases were determined by sigmoidal fitting the experimental results as well as by Hill analysis (Eq. (2); Fig. 3b) and are summarized in Table 1.

$$y = \frac{A_1 - A_2}{1 + (x / x_0)^p} + A_2 \quad (1)$$

where A_1 and A_2 are constant parameters ($A_1 \approx 100$, $A_2 \approx 0$), x is V_{10} concentration in mol/l, x_0 is equal to IC_{50} value and y is enzyme activity (percentage of control).

$$\log\left(\frac{\%\ \text{activity}}{100 - \%\ \text{activity}}\right) = -n \log[I] + n \log IC_{50} \quad (2)$$

where n is Hill's coefficient and $[I]$ is inhibitor (V_{10}) concentration.

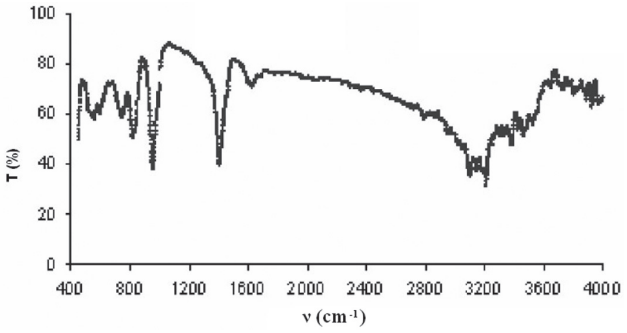

Figure 2. IR spectra (tansmittance (T) dependence on vawenumber (ν)) of $(NH_4)_6V_{10}O_{28}{\cdot}5H_2O$ recorded at room temperature.

All the parameters of both used equations are reported in Table 1. It is clearly apparent that plasma membrane calcium and sodium pumps (SPM ATPases and commercial porcine cerebral cortex) are more sensitive toward V_{10} anion than ecto-ATPase. At the concentration of 1×10^{-5} mol/l V_{10} inhibits both SPM and commercial Na^+/K^+-ATPase as well as Ca^{2+}-ATPase up to 80%, while the effect of the same concentration of V_{10} on the ecto-ATPase activity is negligible. IC_{50} of the enzyme activity is achieved at $(4.74 \pm 1.15) \times 10^{-7}$ mol/l for SPM Na^+/K^+-ATPase, $(1.30 \pm 0.10) \times 10^{-6}$ mol/l for commercial Na^+/K^+-ATPase and $(3.13 \pm 1.70) \times 10^{-8}$ mol/l for Ca^{2+}-ATPase, while the same effect for ecto-ATPase is

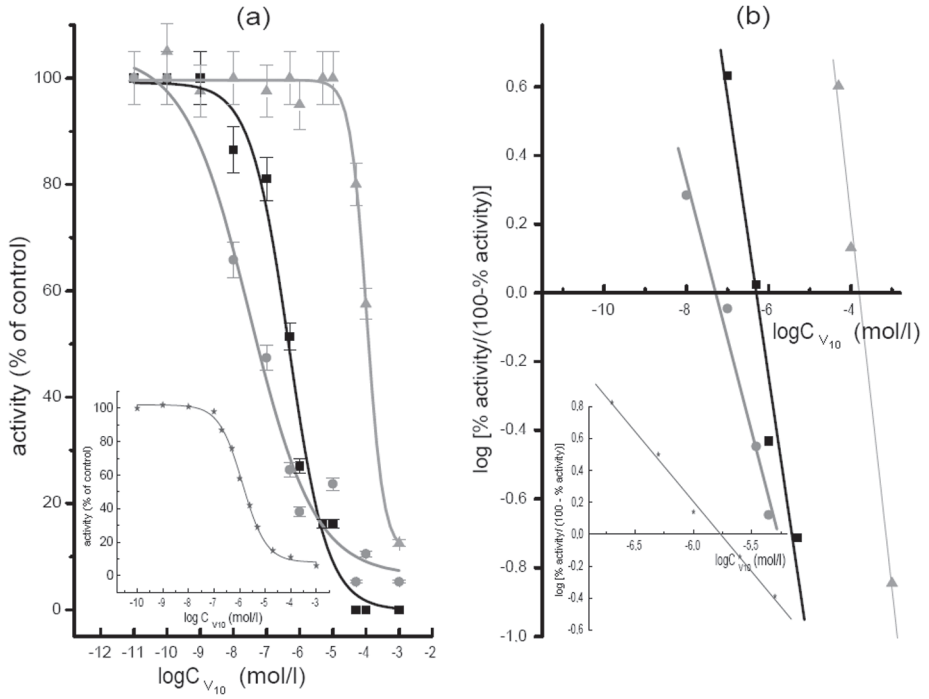

Figure 3. The concentration dependent (a) and Hill analysis (b) inhibition of SPM Na^+/K^+-ATPase (square), Ca^{2+}-ATPase (circle), Mg^{2+}-ATPase (triangle) and commercial porcine cerebral cortex Na^+/K^+-ATPase (inset) by ammonium decavanadate (V_{10}). C_{V10}, concentration of V_{10}.

Table 1. The inhibition parameters of the ATPases inhibition by of ammonium V_{10} obtained by fitting the experimental points by sigmoidal function and Hill analysis

	Sigmoidal fitting			Hill analysis		
	A_1	A_2	p	IC_{50} (x_0) (mol/l)	n	IC_{50} (mol/l)
Na^+/K^+-ATPase	99.11	–0.08	0.75 ± 0.14	$(4.74 \pm 1.15) \times 10^{-7}$	0.81 ± 0.13	4.79×10^{-7}
Ca^{2+}-ATPase	104.59	6.41	0.44 ± 0.11	$(3.13 \pm 1.70) \times 10^{-8}$	0.46 ± 0.05	4.68×10^{-8}
Mg^{2+}-ATPase	99.61	10.85	1.55 ± 0.33	$(1.05 \pm 0.10) \times 10^{-4}$	1.08 ± 0.11	1.58×10^{-4}
Commercial Na^+/K^+-ATPase	101.99	7.94	0.94 ± 0.06	$(1.30 \pm 0.10) \times 10^{-6}$	0.88 ± 0.03	1.71×10^{-6}

A_1, A_2, p – parameters of sigmoidal function; IC_{50}, half-maximum inhibitory concentration of the enzyme activity; IC_{50} (x_0), IC_{50} determined by sigmoidal fitting; n, Hill's coefficiet.

observed at several orders of magnitude higher concentration of V_{10}: $(1.05 \pm 0.10) \times 10^{-4}$ mol/l.

Kinetic analysis

To evaluate the nature of the P-type ATPases inhibition by V_{10}, the kinetic parameters of commercial porcine cerebral cortex Na^+/K^+-ATPase, K_m and V_{max}, were determined by varying the concentration of $MgATP^{2-}$. The kinetic properties of the enzyme were determined in the absence and presence of IC_{50} of V_{10} (1.30×10^{-6} mol/l). The dependence of the initial reaction rate *vs.* substrate concentration in the presence and absence of inhibitor exhibited typical Michaelis-Menten kinetics, and is presented in Fig. 4. Kinetic constants were calculated from the experimental data by nonlinear regression fitting (hyperbola function) as well as Lineweaver-Burk transformation (Fig. 4, inset), and are summarized in Table 2. K_m and V_{max} values obtained by nonlinear regression fitting are in good agreement with values obtained by linear fitting model. As can be seen, V_{10} induced mixed type of inhibition of Na^+/K^+-ATPase activity by significant decreasing its maximal velocity (V_{max} value), as well as decreasing its apparent affinity for ATP (increased K_m value).

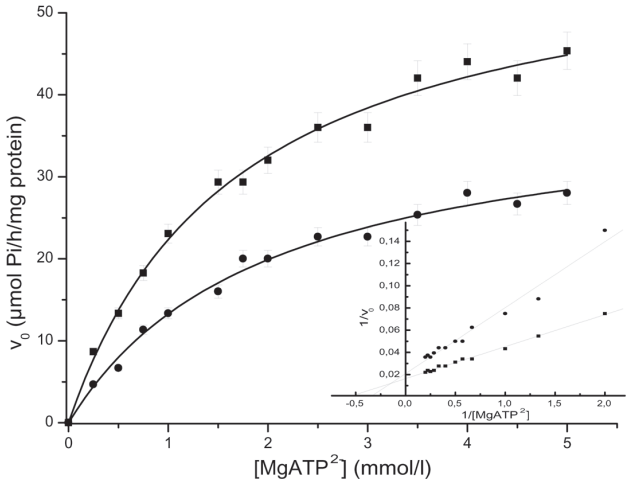

Figure 4. Na^+/K^+-ATPase specific activity (expressed as initial velocity v_0) dependence on $MgATP^{2-}$ in the absence (square) and presence (circle) of 1×10^{-6} mol/l V_{10}. The experimental conditions are described in Materials and Methods. The values (expressed as μmol released inorganic phosphate (Pi)) given are the mean of at least three experiments ± S.E.M., conducted in duplicate. The Lineweaver-Burk transformation of the data is shown in the inset.

Discussion

In the present study we investigated the effect of vanadate decamer on the activity of rat synaptosomal plasma membrane ATPases. Vanadium solution contains different oligomeric (n = 1–10) vanadate species in equilibrium, e.g., monomeric, dimeric, tetrameric and decameric and, with different states of protonation and conformation, depending of the pH (Tiago et al. 2004). The acidification from pH 7 to pH 5 leads to the formation of yellow solution containing the V_{10} core $[V_{10}O_{28}]^{6-}$, which can be found in several protonation states $[H_2V_{10}O_{28}]^{4-}$ $[H_3V_{10}O_{28}]^{3-}$ and $[H_4V_{10}O_{28}]^{2-}$.

Table 2. Kinetic analysis of commercial porcine cerebral cortex Na^+/K^+-ATPase activity in the absence and presence of V_{10} (1.30×10^{-6} mol/l)

	Control	V_{10}
K_m (mmol/l)	1.68 ± 0.14*	2.38 ± 0.20*
	1.76 ± 0.02**	2.69 ± 0.09**
V_{max} (μmol P_i /h/mg)	59.93 ± 2.02*	39.65 ± 1.72*
	61.13 ± 1.75**	41.87 ± 1.35**

V_{10}, decavanadate; K_m, Michaelis constant; V_{max}, maximum enzymatic velocity; Pi, inorganic phosphate; * values obtained by Lineweaver-Burk transformation; ** values obtained by nonlinear regression fitting-hyperbola function.

In this kind of compound, the location of the protons is important because of the basic role played by the oxygen sites in the reactivity of the polyanion. Obtained IR spectra of the synthesized vanadate decamer (Fig. 2) exhibits bands with position characteristic for non-protonated V_{10} anion.

The obtained results (Table 1 and Fig. 3) show that V_{10} exhibits concentration-dependent inhibitory effect on the activity of all investigated enzymes. These results are in agreement with previously reported findings that V_{10} oxoanion inhibits several nucleotide-dependent enzymes (Boyd et al. 1985; Aureliano et al. 2000), although the data about decavanate influence on plasma membrane ATPases are scant.

The sensitivity of investigated ATPases toward vanadate decamer, as shown in our study, is quite different. It is obvious that PMCA is the most sensitive toward V_{10} anion (Table 1). Moreover, obtained inhibitory parameters for V_{10} show that PMCA is two orders of magnitude more sensitive than sarcoplasmic reticulum Ca^{2+}-ATPase from rabbit skeletal muscles (Varga et al. 1985). Both SPM and commercial sodium pumps are about one order of magnitude less sensitive than PMCA, while the activity of ecto-ATPase is about thousand times more resistant to V_{10} anion than investigated P-type ATPases. These results are in agreement with previously reported data, that SPM ecto-ATPase from rat brain is significantly less sensitive toward different metal ions than Na^+/K^+-ATPase (Vasić et al. 1999; Krstić et al. 2005).

Kinetic analysis of the results shows that the presence of V_{10} induces significant decrease in V_{max} and increase in K_m values of Na^+/K^+-ATPase (Table 2) related to control. Moreover, different points of intersection of straight lines with x-axis and y-axis obtained from Lineweaver-Burk analysis of the kinetic data in the absence and presence of V_{10} confirm mixed mode of interaction (Fig. 4, inset). It indicates that V_{10} as a reversible mixed inhibitor of Na^+/K^+-ATPase activity binds to enzyme sites participating in both substrate binding and catalysis. Obtained mixed inhibition is in agreement with previously reported results that there are two binding sites for V_{10} on Ca^{2+}-ATPase which belongs, as well as Na^+/K^+-ATPase, to P-type of ATPases (Varga et al. 1985; Stokes et al. 2005). Decrease in apparent affinity for $MgATP^{2-}$ is in agreement with competition of V_{10} with fluorescein isothocyanate for ATP binding site of Ca^{2+}-ATPase (Csermely et al. 1985). Obtained decrease in maximal velocity of the enzyme reaction probably can be explained by binding of V_{10} to another binding site that is, as earlier reported (Csermely et al. 1985), binding site for inorganic phosphate on Ca^{2+}-ATPase.

It could be summarized that the obtained dose-dependent inhibition of PMCA and sodium pump by V_{10}, as well as kinetic analysis, is in agreement with previously reported findings that decameric vanadate species block the active side of P-type ATPases and consequently affect phosphorylation step in the enzyme cycle of P-type ATPases (sodium and calcium pump) (Stokes et al. 2005). However, this mechanism could not be responsible for obtained ecto-ATPase inhibition (a member of ecto-NTPDases) and probably occurs *via* different mechanism resulting in less sensitivity compared to P-type ATPases (sodium and calcium pump).

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