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THIACALIX[4]ARENE C-1193 – A PROMISING INHIBITOR OF THE SODIUM PUMP IN THE UTERINE SMOOTH MUSCLE CELLS

O. V. MALIUK[1]✉, T. O. VEKLICH[1], O. V. TSYMBALYUK[2], O. V. BEVZA[1], S. O. CHERENOK[3], A. I. SELIKHOVA[3], V. I. KALCHENKO[3], S. O. KOSTERIN[1]

[1]*Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine, Kyiv, Ukraine;*
✉*e-mail: sanya2000ua@gmail.com;*
[2]*Educational and Scientific Institute of High Technologies, Taras Shevchenko National University of Kyiv, Kyiv, Ukraine;*
[3]*Institute of Organic Chemistry, National Academy of Sciences of Ukraine, Kyiv, Ukraine*

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Thiacalix[4]arene C-1193 (25,27-dibutoxythiacalix[4]arene-bis-hydroxymethylphosphonic acid) was shown to inhibit the activity of Na^+,K^+-ATPase with a high efficiency ($I_{0.5}$ = 42.1 ± 0.6 nM) with no effect on the activity of Mg^{2+}-ATPase, Ca^{2+}-ATPase and Ca^{2+},Mg^{2+}-ATPase in the plasma membrane fraction of rat uterine smooth muscle cells. The kinetic regularities of the C-1193 inhibitory effect on Na^+,K^+-ATPase activity were investigated. It was demonstrated that C-1193 increased the enzyme activation constant by Na^+ but not by K^+ ions. The contractile activity of the rat uterine horns was investigated by tenzometric methods with the use of longitudinal uterine smooth muscle strips with intact endometrium. C-1193 induced a considerable increase in the amplitude of the acetylcholine-induced contractions as well as the maximal velocity of the contraction and relaxation phases. No effect of C-1193 on contractive activity induced by the selective agonist of M_3-cholinoreceptors cevimeline was observed. The results of computer simulation showed that C-1193inhibitory effect must be related to the cooperative action of methylene bisphosphonate fragments on the upper rim of the calixarene platform, and the linker sulfur atoms of calixarene "cup" on the Na^+,K^+-ATPase macrostructure.

K e y w o r d s: thiacalix[4]arenes, Na^+,K^+-ATPase, plasma membrane, smooth muscle cell, myometrium, computer simulation, docking.

Mg^{2+},Na^+,K^+-ATP-dependent adenosine triphosphatase (sodium pump) is an electrogenic Ca^{2+}-independent system of active ion transporting which implements the transfer of univalent ions of Na and K between the extracellular space and cytosol via the plasma membrane (PM). The regulation of the cellular exchange, the ensuring of electric excitability of the nervous and muscle tissues, and other biochemical and physiological functions are performed due to the balance of the electrochemical gradients, which, in its turn, is supported by the work of the abovementioned transporting enzyme [1-5].

It was determined that the impairment of calcium homeostasis in the excitable tissues is often a reason for pathologies, occurring in the systems of hollow organs. In particular, these are the impairments of digestion processes, the complications of labor, the problems in the work of cardiovascular, genitourinary, and other systems of the organism. It is also known that the diagnosed diabetes and ischemia are notable for a decrease in the enzymatic activity of the sodium pump, which surely plays an important role in supporting the homeostasis of free calcium [1]. Therefore, the search for reversible selective and affine inhibitors and activators with the ability to have a targeted action on Na^+,K^+-ATPase to correct its work is a promising perspective both for the fundamental determination of the mechanisms of pharmaco- and electromechanical conjugation and investigation of ion transport and signaling pathways and for practical application in the elaboration of pharmaceutical preparations, the scheme of whose synthesis may be based on the data obtained.

In this context, macrocyclic nanocompounds from the class of calix[4]arenes may be considered

rather promising. They are synthesized via cyclocondensation of para-substituted phenols and formaldehyde, characterized by low toxicity [6, 7] and immunogenicity [8, 9], and some compounds of this class demonstrate antiviral, antibacterial, antithrombotic, and antitumour properties. Some of them have membranotropic action and are capable of penetrating the sarcoplasm via PM and of reverse modification of the functional activity of some proteins [10, 11]. Thus, these nanocompounds are suggested for consideration as potential target effectors to solve the abovementioned tasks.

Our previous investigations, conducted using the PM preparation of uterine myocytes, demonstrated that thiacalix[4]arene C-1193 (the titer is stated) is efficient in inhibiting the enzymatic activity of ouabain-sensitive Na^+,K^+-ATPase, practically not impacting the activities of Mg^{2+}-ATPase, Ca^{2+}-ATPase and Ca^{2+},Mg^{2+}-ATPase of the PM [12].

This study aimed to investigate the kinetic regularities and the mechanism of the inhibitory action of thiacalix[4]arene C-1193 on Na^+,K^+-ATPase of the plasma membrane of the myometrium cells.

Materials and Methods

A. The structure and synthesis of thiacalix[4]arene C-1193. Thiacalix[4]arene C-1193 (25,27-dibutoxythiacalix[4]arene-bis-hydroxymethylphosphonic acid) (Fig. 1) was synthesized and characterized using the NMR method at the Department of the Chemistry of Macrocyclic Compounds at the Institute of Organic Chemistry, the NAS of Ukraine (the department is headed by Prof. V.I. Kalchenko, the full member of the NASU). The synthesis method for the mentioned thiacalix[4]arene was described previously [12].

B. Biochemical studies. The biochemical studies were conducted at the Muscle Biochemistry Department of the Palladin Institute of Biochemistry, the NAS of Ukraine (headed by S. O. Kosterin, the full member of the NAS of Ukraine).

Preparative biochemistry. The PM fraction of uterine smooth muscle cells was isolated from the porcine myometrium as described before [13, 14].

The protein content in the membrane fraction was determined by the method of M. Bredford [15] using the reaction with Coomassie reagent – G250.

Enzymological studies. The "total" Mg^{2+},Na^+,K^+-ATPase activity was determined in the PM fraction of myometrium cells as described before [13], at 37°C in the standard medium (the volume of 0.4 ml), containing (mM): 1 ATP, 3 $MgCl_2$, 125 NaCl, 25 KCl, 1 EDTA, 20 Hepes-tris-buffer (pH 7.4), 1 NaN_3, 0.1 μM thapsigargin and 0.1% digitonin. The amount of membrane fraction protein in the probe was 20–30 μg. The incubation time – 4 min. The enzymatic reaction was initiated by the introduction of the aliquote (50 μl) of PM suspension to the incubation medium and terminated by the introduction of 1 ml of the "stop"-solution to the incubation mixture as follows: 1.5 M acid sodium acetate, 3.7% formaldehyde, 14% ethanol, 5% TCA, pH 4.3 (at 8°C). The presence of Ca^{2+}-chelating agent of EDTA in the incubation medium ensured the binding of endogenous ions of Ca therein.

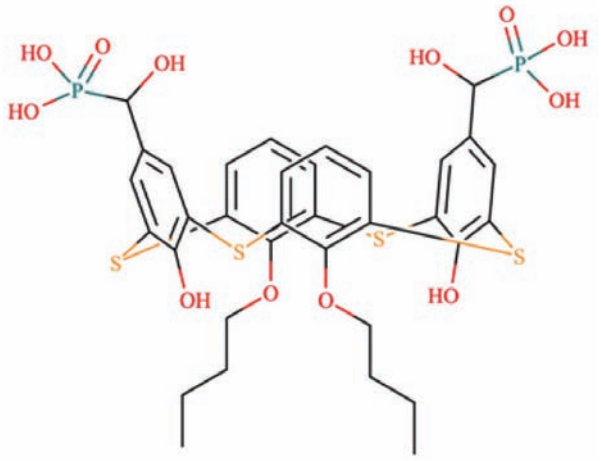

Fig. 1. The structural formula of thiacalix[4]arene C-1193

The "ouabain-sensitive" Na^+,K^+-ATPase activity was estimated by the difference between the values of the "total" ATPase activity in the presence and absence of 1 mM of ouabain (selective inhibitor of Na^+,K^+-ATPase [16]).

The relative enzymatic activity of Na^+,K^+-ATPase in the sarcolemma of the porcine myometrium was 10.2 ± 0.7 μmol P_i/mg of protein per one hour respectively (n = 7) [13].

The amount of P_i reaction was determined by the method of W. Rathbun et V. Betlach [17].

In the experiments on investigating the action of different ratios of the concentrations $[Na^+]/[K^+]$ (on condition of a stable total concentration of univalent cations $[Na^+] + [K^+]$ = 150 mM) and thiacalix[4]arene C-1193 (10–100 nM) on Na^+,K^+-ATPase activity, we used the standard incubation medium, described above, which was supplemented with the aliquote of NaCl and KCl solution in the corresponding ratio

of concentrations. All the experiments involved the use of the concentrated (1 mM) solution of thiacalix[4]arene C-1193 in DMSO, which was further diluted with water till the required final concentration.

Kinetic estimates. The kinetic parameters of the action of Na and K ions on the enzymatic activity of Na^+,K^+-ATPase were estimated using the concentration dependencies, built in double logarithmic coordinates according to the linearized equation of Hill, $lg[(V_{max} - V)/V] = n_H \cdot lgK - n_H \cdot lgS$, where V – relative enzymatic activity, V_{max} – maximal relative enzymatic activity, K – apparent constant of the activation with Na or K ions, S – concentration of the substrate or ion-activator in the incubation medium. In case of such charts, the typical value of the mean square deviation of the approximation coefficient was 0.9–0.99. The kinetic and statistical calculations were done using the MS Excel software.

Tenzometric experiments. The contractive activity of the rat uterine horns was investigated by tenzometric methods in the isotonic mode using the preparations of the longitudinal smooth muscles (2×10 mm) with the intact endothelium.

All the preparative procedures were conducted in the Krebs solution of the following composition (mM): NaCl – 120.4; KCl – 5.9; $NaHCO_3$ – 15.5; NaH_2PO_4 – 1.2; $MgCl_2$ – 1.2; $CaCl_2$ – 2.5; glucose – 11.5 (pH 7.4).

The preparations of smooth muscles were placed into the working chamber of tenzometric equipment with the flowing Krebs solution (the flow rate of 5 ml/min, thermostated at 37.5 ± 0.3°C), given a fixed load of 10 mN, and left for 1 h until the occurrence of spontaneous contractions with constant amplitude and frequency.

Calix[4]arene C-1193 was preliminarily dissolved in DMSO and administered to the solutions in the concentration of 10^{-5} M (DMSO concentration was 0.1%); the control contractions were registered on the background of 0.1% DMSO.

The following activators of contractions were used in the study: the solutions of a non-selective agonist of muscarinic choline receptors, neuromediator acetylcholine, and a selective agonist of muscarinic choline receptors of M3-subtype, cevimeline (Sigma) which were used in the concentrations of 10 and 100 μM, respectively.

The kinetic analysis of the induced muscle contractions was performed by the method of Kosterin-Burdyga with the estimation of normalized maximal velocities of the phases of contraction (V_{nc}) and relaxation (V_{nr}) [18].

Computer simulation. The three-dimensional structure of thiacalix[4]arene C-1193 was created and its geometry was optimized by the method of energy minimization, which was conducted by the MMFF94 program (Merck Molecular Force Field), with the consideration of electrostatic and van der Waals interactions under the following conditions: ascension slope – 100; step size of ascension slope – 0.002 nm; conjugated gradient of ascension steps –1 0; size of conjugated gradient of ascension steps – 0.002 nm. The optimized structures were used while docking with the enzyme in AutoDock program, version 4.2 [19].

The optimization of the geometry (energy minimization) of the interaction between thiacalix[4]arene and $Na^+,K+$-ATPase, and the molecular dynamics was done in the Chimera program [20]. The optimal results were selected both by geometric indices for the ligand position in the center of binding the complex "calixarene – Na^+,K^+-ATPase" and by the energy indices, using the functions of estimating the binding energy in the "receptor-ligand" complex, "built" into the docking program. Chimera program was used to analyze the results and to prepare the figures using the simulation results.

The stability of binding and the conformational changes in the investigated complex were studied by the method of molecular dynamics (MD) with the consideration of electrostatic and Lennard-Jones interactions under the following conditions: interval of a step of calculation – 1.0 fs; frame interval – 10 fs; ending after 10,000 steps; velocity of heating/cooling – 1 ccal/atom/ps; Andersen barostat – 1.0132 bar, deoxidation time – 1.5 ps; Nose thermostat – 298 K, deoxidation time – 0.2 K/ps. The field of force Amber ff14SB was used.

The spatial structure of Na^+,K^+-ATPase of PM with 3a3y identifier in the database of these proteins (RSCB Protein Data Bank) was used in the study.

Statistical analysis. The statistical analysis of the data obtained was conducted by standard methods using Student's *t*-criterion. The kinetic and statistical calculations were done using the MS Excel software.

Reagents. The following reagents were used in the experiments: ATP, Hepes, ouabain, thapsigargin (Sigma, USA), tris-hydroxymethyl-aminomethane (Reanal, Hungary), digitonin (Merck, Germany), EDTA (Fluka, Switzerland), oxytocin (Gedeon Richter, Hungary). Other reagents were analytically and chemically pure, produced in Ukraine.

Results and Discussion

In our previous studies, we demonstrated that a synthetic substance, thiacalix[4]arene C-1193 (25,27-dibutoxythiacalix[4]arene-bis-hydroxymethylphosphonic acid) in the concentration of 100 μM effectively (by 94% as compared against the control) inhibited the activity of Na^+,K^+-ATPase of the PM of uterine myocytes (Fig. 2, *A*). At the same time, this substance, used in the same concentration, practically did not impact the enzymatic properties of the "basal" Mg^{2+}-ATPase, Ca^{2+}-ATPase and Ca^{2+},Mg^{2+}-ATPase of PM: the corresponding activities were 97.1 ± 0.8, 95.2 ± 0.9, and 93.1 ± 1.1% regarding the control value (Fig. 2, *A*).

Therefore, thiacalix[4]arene C-1193 selectively (at the PM level) inhibits the activity of Na^+,K^+-ATPase of PM, not affecting the activities of Ca^{2+},Mg^{2+}-ATPase, Mg^{2+}-ATPase and Ca^{2+}-ATPase of PM.

In our further investigations, we studied the concentration dependence of the inhibitory action of thiacalix[4]arene C-1193 (10^{-8}–10^{-4} M) on the activity of Na^+,K^+-ATPase of PM (Fig. 2, *B*). The estimated value of the inhibition coefficient $I_{0.5}$ is 42.1 ± 0.6 nM, the value of the Hill coefficient n_H is 0.36 ± 0.05.

The experiment results demonstrate that the investigated thiacalix[4]arene C-1193 with high efficiency ($I_{0.5}$ = 42.1 ± 0.6 nM) inhibits the enzymatic activity of Na^+,K^+-ATPase of PM, at the same time, it does not affect the activities of Mg^{2+}-ATPase, Ca^{2+}-ATPase and Ca^{2+},Mg^{2+}-ATPase of PM (Fig. 2).

Therefore, for further kinetic interpretation of the action of thiacalix[4]arene C-1193 on the enzymatic activity of Na^+,K^+-ATPase of PM of the myometrium we investigated its action on the nature of concentration dependencies of this activity on the concentration ratios $[Na^+]/[K^+]$.

The chart of dependence of the enzymatic activity of Na^+,K^+-ATPase on the concentration ratios $[Na^+]/[K^+]$ (under condition of stable total concentration of univalent cations $[Na^+] + [K^+]$ = 150 mM) has a typical dome-like form (the left and right branches – "sodium" and "potassium" ones, respectively); in case of the absence of Na^+ or K^+ ions in the incubation medium, the enzymatic activity of Na^+,K^+-ATPase is not tested at all (Fig. 3), which is characteristic of this enzyme [21].

Thus, under these conditions, the optimal ratio of concentrations $[Na^+]/[K^+]$ for the functioning of Na^+,K^+-ATPase is 115–125 mM NaCl and 35–25 mM KCl. The activation constant value K_{Na+} for Na ions for ouabain-sensitive Na^+,K^+-ATPase is 52.7 ± 2.0 mM ("sodium branch" of the control chart in Fig. 3) (Fig. 4, *A*). The value of the activation constant K_{K+} for K ions is 9.2 ± 0.5 mM ("potassium branch" of the control chart in Fig. 3) (Fig. 4, *B*).

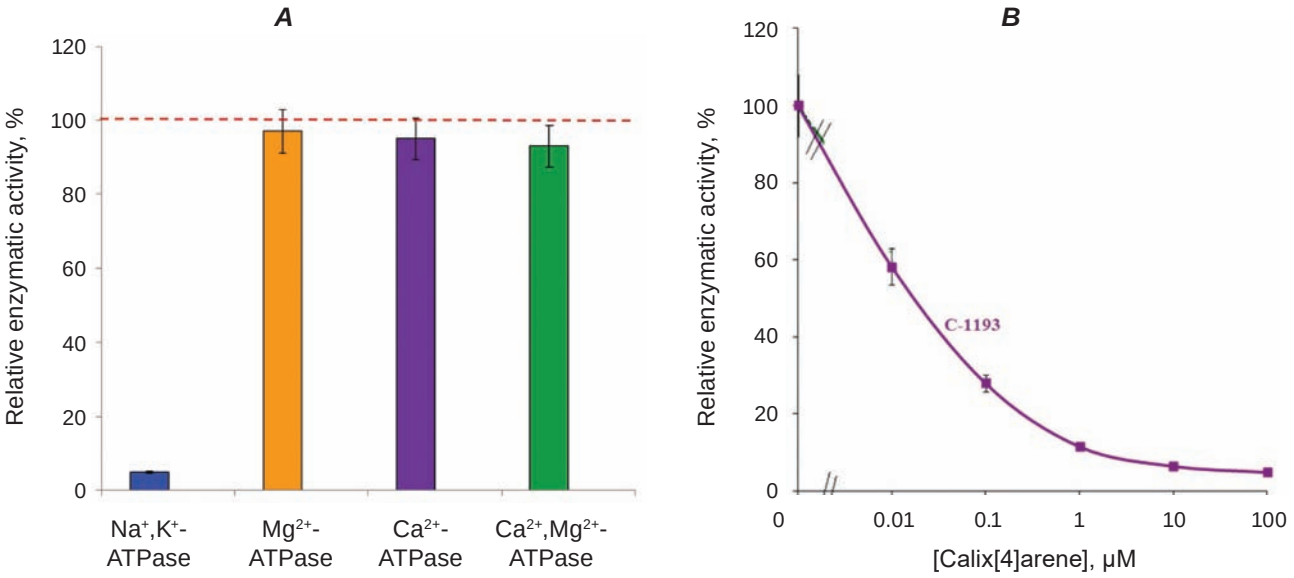

Fig. 2. ***A*** *– The effect of thiacalix[4]arene C-1193 (100 μM) on ATP-hydrolase activities in plasma membranes of the myometrium cells (M ± m, n = 5).* ***B*** *– The concentration dependence of the action of thiacalix[4]arene C-1193 on the activity of Na^+,K^+-ATPase of plasma membranes (M ± m, n = 5). The values of relative enzymatic activities in the absence of thiacalix[4]arene C-1193 in the incubation medium are accepted as 100%*

We studied the impact of thiacalix[4]arene C-1193 on the enzyme affinity to Na and K ions and investigated the action of five concentrations of thiacalix[4]arene C-1193 (10, 25, 50, 75, and 100 nM, respectively) on the dependence of Na^+,K^+-ATPase activity of the uterine smooth muscle cells on the concentration of Na and K ions (Fig. 3). In all the cases, there was a monotonous decrease in the activity of Na^+,K^+-ATPase, and the dependence of the enzymatic activity on the concentration ratio $[Na^+]/[K^+]$ demonstrated the character, similar to the corresponding control dependence without thiacalix[4]arene C-1193, but there is a decrease in the plateau level of activity with the increase in the concentration of thiacalix[4]arene.

Using the obtained dependencies, we estimated the kinetic parameters of the activation of Na^+,K^+-ATPase by Na and K ions and determined the dependence of these characteristics on the thiacalix[4]arene C-1193 concentration in the range from 10 to 100 nM. We demonstrated that thiacalix[4]arene C-1193 (in the concentrations of 75 and 100 nM) under isotonic conditions reliably increased the value of the activation constant K_{Na+} by Na ions of the ouabain-sensitive Na^+,K^+-ATPase: from 52.7 ± 2.1 mM (control) to 75.3 ± 4.3 mM (in the presence of thiacalix[4]arene C-1193) (Fig. 4, *A*). At the same time, there were no changes in the affinity of the mentioned enzyme to K ions – the activation constant value for these ions of K_{K+} was 9.2 ± 0.5 mM (control) and 9.4 ± 0.8 mM (in the presence of 100 nM of thiacalix[4]arene C-1193) (Fig. 4, *B*). The estimated values of Hill coefficients n_H of their action reliably did not depend on the concentration of thiacalix[4]arene C-1193 (Fig. 4, *A* and *B*).

Therefore, it is possible to assume that one of the reasons for the inhibitory action of thiacalix[4]arene C-1193 on Na^+,K^+-ATPase may be related to the decrease in the affinity of the enzyme to Na ions under the effect of this substance.

Acetylcholine, a neuromediator of the parasympathetic nervous system, is a relevant physiological regulator of the tone and contractive activity of uterine smooth muscles. In the myometrium tissue, acetylcholine acts as an excitation factor, and indirectly activates the muscarinic choline receptors of M_2 and M_3-subtypes [22, 23]. Thus, we used the model of pluricellular preparations of the longitudinal smooth muscle stripes of uterine horns with preserved endo-

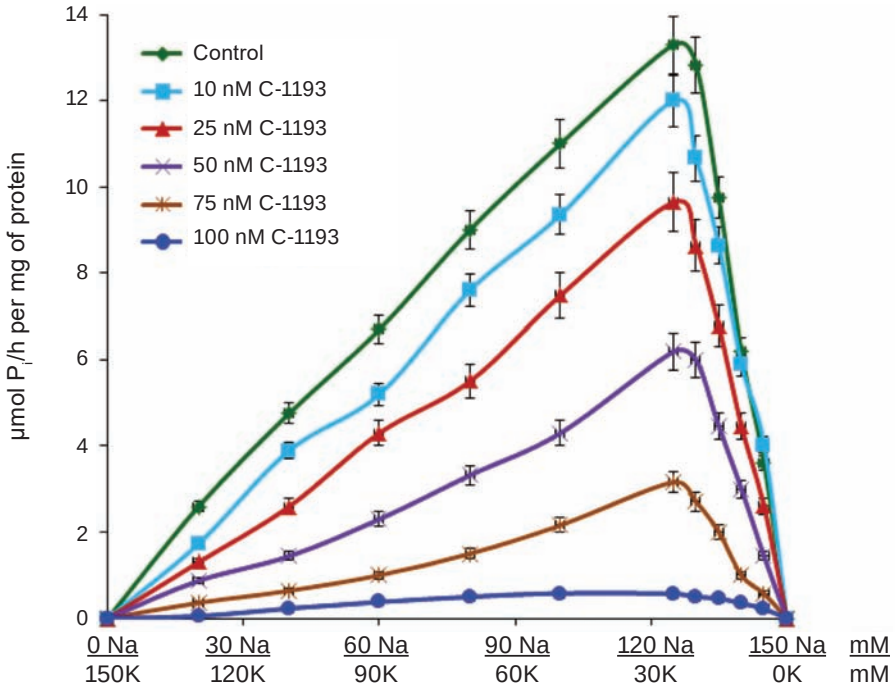

Fig. 3. The impact of the increase in the concentration of thiacalix[4]arene C-1193 on the dependence of the relative activity of Na^+,K^+-ATPase in the PM fraction of the myometrium cells on the $[Na^+]/[K^+]$ ratio (M ± m, n = 5). The experiments were conducted on condition of preserving the isotonicity of the incubation medium: $[Na^+] + [K^+] = 150$ mM

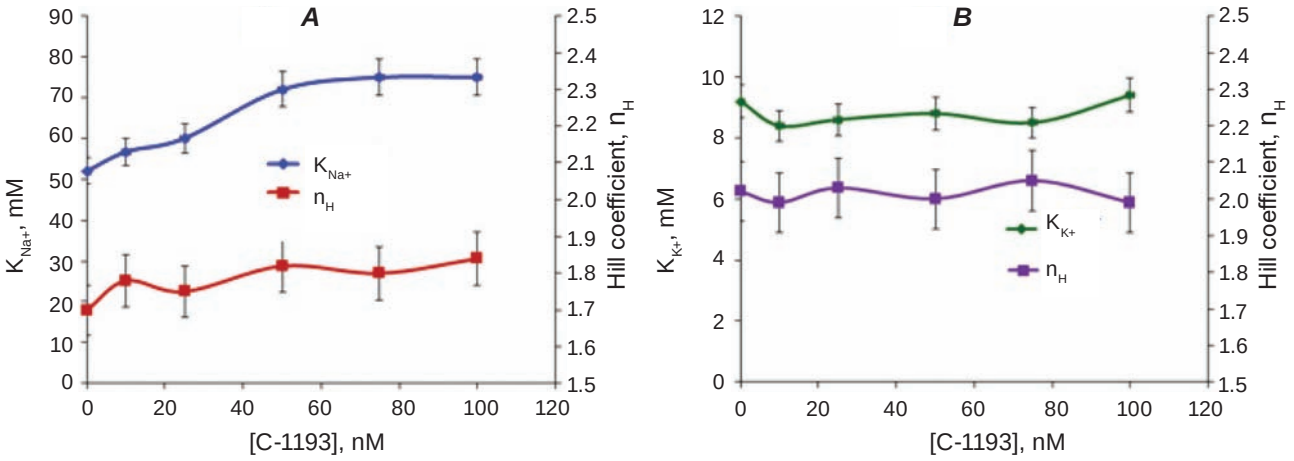

*Fig. 4. The action of thiacalix[4]arene C-1193 on the kinetic parameters of the dependence of the activity of Na^+,K^+-ATPase in PM fraction of the myometrium cells on the concentration of Na (**A**) and K (**B**) ions ($M \pm m$, $n = 5$)*

metrium and studied the action of thiacalix[4]arene C-1193 on the acetylcholine-activated isotonic contractions.

It was determined that the pre-incubation (for 30 min) of the myometrium preparations with thiacalix[4]arene C-1193 (10 μM) caused a considerable increase in the amplitude of acetylcholine-induced (10 μM) contractions (Fig. 5, *A*) on average to 121.2 ± 6.6% ($n = 5$, $P < 0.05$). A typical trend of the contractile response of the myometrium under the action of C-1193 with increased contraction amplitude and altered kinetics of contraction and relaxation processes is shown in Fig. 5, *A*. The mechanokinetic analysis was used to determine that this inhibitor of Na^+,K^+-ATPase of the plasma membrane also caused a considerable increase in the normalized maximal velocities of the phases of contraction (Vn_C) and relaxation (Vn_R): to 183.2 ± 6.6% ($n = 5$, $P < 0.01$) and 221.2 ± 8.6% ($n = 5$, $P < 0.01$), respectively (Fig. 5, *B*).

The amplitude of acetylcholine-induced contractions of myometrium is considerably conditioned by the stimulation of the receptors of M_3-subtype, conjugated with $G_{q/11}$-proteins, which leads to the secondary messenger, inositol-1,4,5-triphosphate (ITP) and ITP-dependent release of Ca^{2+} from the sarcoplasmic reticulum [24, 25]; a significant contribution into the level of acetylcholine-induced contractions is also made by Ca^{2+} ions which enter the myoplasm from the extracellular environment. The relaxation process and the tonic component of acetylcholine-induced contractions result from the superposition of several processes: the activation of the receptors of M_2-subtype, conjugated with $G_{i/o}$-proteins, and the Ca^{2+} release from the extracellular environment, the sensitization of the proteins of the contractive apparatus to Ca^{2+}, and the extrusion of these cations from the myoplasm [26, 27].

To check our assumption of the ability of C-1193 to stimulate the phase component of the acetylcholine-induced contractions due to selective stimulation of the receptors of M_3-subtype, on the following stage, we investigated the action of thiacalix[4]arene C-1193 on the contraction of myometrium preparations, induced by the application of the selective agonist of M_3-cholinoreceptors, cevimeline (100 μM).

It was found that thiacalix[4]arene C-1193 did not have a considerable effect on the amplitude of the cevimeline-induced contractions, but it slowed their relaxation down (Fig. 6, *A*).

These effects of C-1193 reflected on the parameters of the normalized maximal velocities of contractions: the velocity of the contraction phase (Vn_C) was at the control level (on average, 99.0 ± 4.8%, $n = 5$, $P > 0.05$), while the velocity of the relaxation phase reliably decreased (on average, down to 62.6 ± 5.1%, $n = 5$, $P < 0.01$) (Fig. 6, *B*). Thus, the increase in the amplitude of acetylcholine-induced contractions cannot be explained by the selective activation of the processes of conjugating excitation and contraction via M_3-subtype receptors. The effects of the activation of acetylcholine-induced contractions by thiacalix[4]arene C-1193 may be the result of the increased

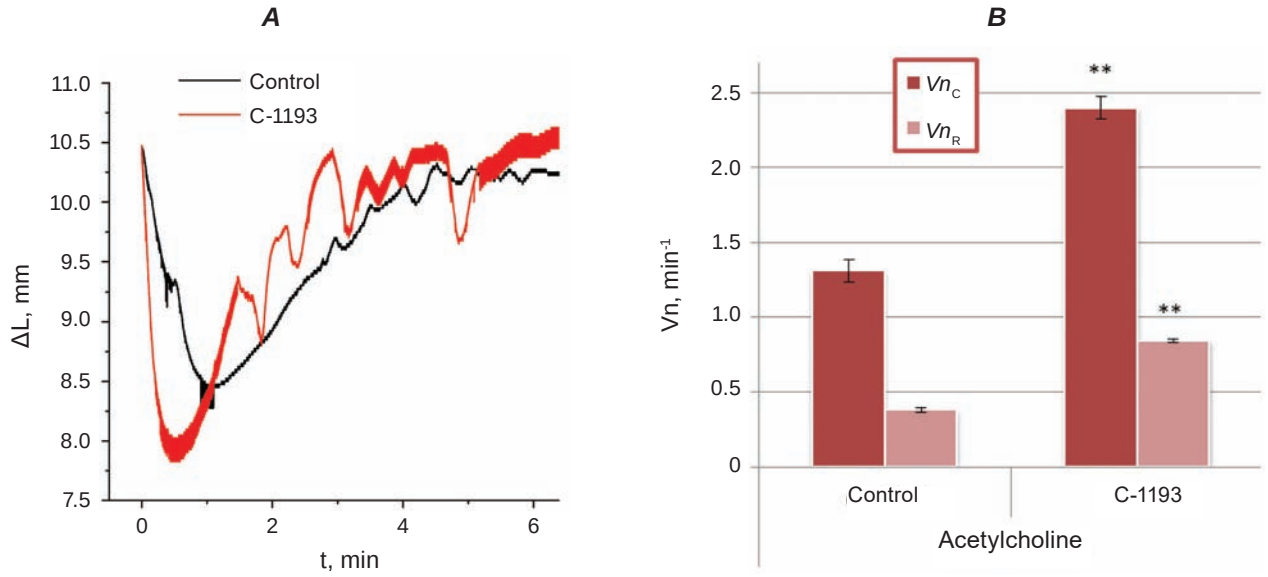

*Fig. 5. The acetylcholine-induced (10 μM) isotonic contractions of the longitudinal smooth muscles of rat uterine horns in control and against the background of thiacalix[4]arene C-1193 (10 μM): **A** – typical mechanograms; **B** – normalized maximal velocities of the phases of contraction (Vn_C) and relaxation (Vn_R), n = 5, **P < 0.01*

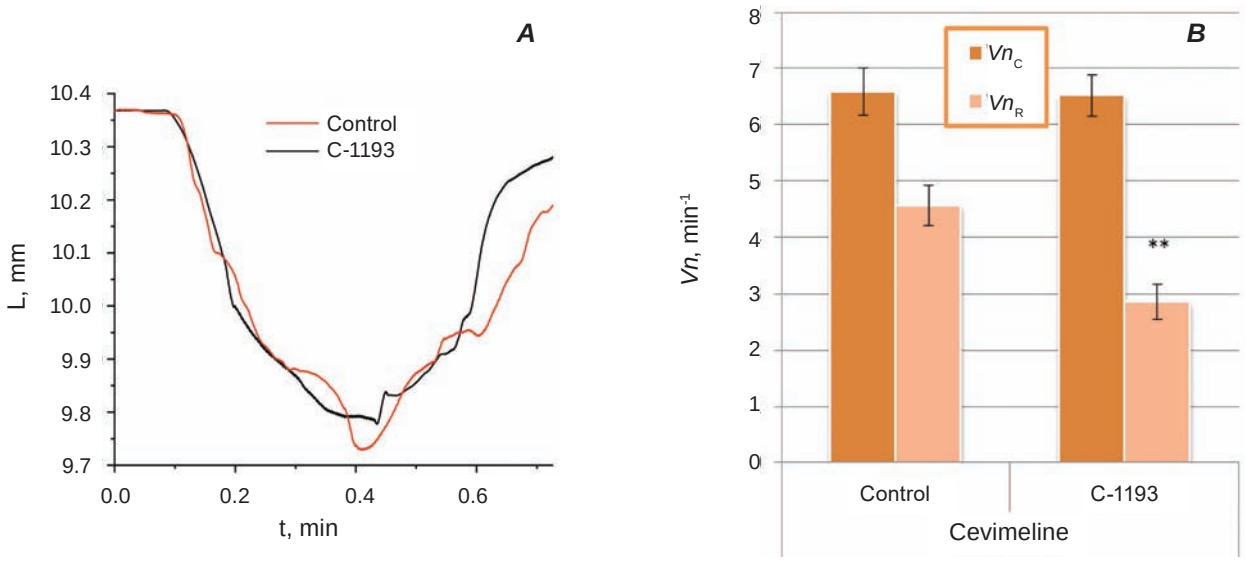

*Fig. 6. The cevimeline-induced (100 μM) isotonic contractions of the longitudinal smooth muscles of rat uterine horns in control and against the background of thiacalix[4]arene C-1193 (10 μM): **A** – typical mechanograms; **B** – normalized maximal velocities of the phases of contraction (Vn_C) and relaxation (Vn_R), n = 5, **P < 0.01*

level of Ca^{2+} in the cytoplasm due to the impaired work of Na^+-dependent secondary active transport of myocytes, in particular, the transition of the functioning of Na^+,Ca^{2+}-exchanger of the plasma membrane into the reverse mode [28, 29].

Acetylcholine as a neurotransmitter of the parasympathetic nervous system is an important factor in the regulation of the excitability of uterine smooth muscles. As noted in the work of A. Choppin [30], muscarinic cholinergic receptors of subtypes M_2 and M_3 are expressed in the rat myometrium in a ratio of 1/3 : 2/3. According to the studies of F.M.F. Abdalla [23], the functional contribution of individual subtypes of these receptors significantly outweighs the

M_3 subtype. In particular, F.M.F. Abdalla established this by registering carbacholine-induced contractions of the rat myometrium against the background of cholinergic receptor antagonists with different selectivity to receptor subtypes (atropine, PfHHSiD, 4-DAMP and metocramine).

Also in favor of the conclusion about the predominant contribution of the M_3 receptor subtype to acetylcholine-induced myometrial contractions is evidenced by comparative functional studies conducted on wild-type mice, with knockout of M_2 and M_3 receptors separately, as well as with simultaneous knockout of both of these receptors [25]. In particular, all used muscarinic cholinergic receptor antagonists AF-DX116, 4-DAMP, pF-HHSiD, gimbacin, methoctramine, pirenzepine and tropicamide on the myometrium of control animals acted as competitive antagonists with pKb parameters similar to the pKi of these M_3 receptor antagonists. In the case of animals with knockout of M_2 receptors, carbachol caused contractile responses significantly weaker than in controls and only at concentrations of $5 \cdot 10^{-6}$ M and higher; at the same time, when the M_3 receptor subtype was knocked out, carbacholine did not cause any contractile responses. At present, the most likely hypothesis regarding the role of individual subtypes of muscarinic cholinergic receptors in uterine smooth muscle is the mediated enhancement of the excitatory effects of M_3 cholinergic receptor activation through M_2 subtype cholinergic receptors.

As has been shown in the longitudinal smooth muscle tissue of the uterus of mice [31], acetylcholine-induced contractions are enhanced against the background of blocking butyric cholinesterase (which is the dominant cholinesterase in longitudinal muscle tissue), so we cannot also exclude that a decrease in cholinesterase activity may contribute to the effect of enhancing acetylcholine-induced myometrial contractions in the presence of calix[4]arene C-1193.

In myometrial tissue, α- and β-adrenoceptors are expressed, with β-adrenoceptors significantly predominating in the longitudinal muscle layer, accounting for about 85% [32]. Activation of the latter receptors leads to an increase in adenylate cyclase activity in a Gs-dependent manner, while cAMP, through the activation of protein kinase A, causes relaxation of the uterine muscles mainly due to inhibitory phosphorylation of myosin light chain kinase [33]; this effect is used in particular in clinical practice for the treatment of premature labor. It is important to note that the sympathetic nervous system exhibits physiological antagonism to the effects of cholinergic receptor activation in uterine smooth muscle. As was established in mouse uterine smooth muscle, norepinephrine, the nonselective β-adrenoceptor agonist isoprenaline, and the adenylate cyclase activator forskolin inhibited carbachol-activated contractions [34]. Therefore, we cannot exclude that the enhancement of acetylcholine contractions under the action of calix[4]arene C-1193 may be due to a decrease in adenylate cyclase activity in uterine myocytes.

C-1193 is a sulfur-containing analogue of the previously described and investigated calix[4]arene C-99. Due to four linker atoms of sulfur in the composition of the macrocyclic platform, thiacalix[4]arene C-1193 has a large size of the macrocycle as compared to its predecessor (C-99) and a higher reactive ability.

More detailed data about the spatial structure of thiacalix[4]arene C-1193 was obtained by computer simulation using MMFF94 method, and the total energy after the energy minimization was 89.1 ccal/mol.

In the models, obtained after the minimization, the benzol rings with phosphonate residue are almost parallel, and the para-non-substituted benzol rings are almost orthogonal to the main plane of the macrocycle, formed by the linker atoms of carbon or sulfur, thus, they are spatially available for intermolecular interactions.

Here, the geometrical parameters of energetically minimized structures of C-99 and C-1193 are as follows: the distances between phosphorus atoms – 0.5 and 0.4 nm, respectively, the distance between the diametrical linker atoms of carbon and sulfur – 0.7 and 0.8 nm, respectively. Therefore, the replacement of methylene linker atoms in the macrocyclic platform C-99 with sulfur atoms (molecule C-1193) changes the geometric parameters of the macrocycle and may impact the biological activity of the molecule.

The distance between the adjacent atoms of oxygen on the lower rim of the macrocycle, two proximal pairs of propoxy residues is 0.31 nm, i.e. they may form intramolecular hydrogen bonds with adjacent propoxy groups, which stabilize this conformation of calixarene.

We determined the potential sites of interaction between ligands and ligand-binding sites (LBS) of Na^+,K^+-ATPase, the presence of which was con-

firmed by the values of the minimal binding energy and the aminoacid environment of the LBS. The types of interactions between the enzyme and thiacalix[4]arene C-1193 were also determined.

The exact localization of C-1193 in the LBS of the enzyme was complicated due to the fact that Na^+,K^+-ATPase is a membrane-bound enzyme, and the scientific literature contains data about rather considerable shifts of cytoplasmic domains of ATPases of P-type regarding each other in the enzymatic cycle. Thus, we determined the most probable regions of interaction between the calixarene and the enzyme, confirmed by the values of minimal binding energy and aminoacid environment of the LBS.

Taking into consideration the structural specificities of thiacalix[4]arene C-1193, we analyzed the data about the binding of these ligands, obtained by the docking method, to determine the mechanism of their action of the enzyme work. We defined possible places of binding ("cavities") of the investigated ligands to the LBS of Na^+,K^+-ATPase and studied their interaction with the receptor, then selected a number of complexes with the least total energy.

Thus, the determination of the most probable sites of thiacalix[4]arene C-1193 binding to Na^+,K^+-ATPase demonstrated that it may form a complex with the enzyme in two most probable regions (Fig. 7).

It was shown that C-1193 is very likely to form a complex with the enzyme in the region 1 (between N- and A-domains of Na^+,K^+-ATPase), close to the saline bridge between Arg551 in N-region and Glu223 in A-domain. Therefore, the interaction between C-1193 and aminoacid residues in this region may affect the location of the substrate and the hydrolysis of the covalent enzyme-substrate intermediate. It was found that the interaction with the phosphonate groups of calixarene may involve residues Lys212.A, Arg240.A and Arg551.A. Linker atoms of sulfur of the calixarene "cup" interact with the residues Glu223.A and Glu550.A (in the names of aminoacid residues, A means α-domain, and B – β-domain of the enzyme, respectively). Calix[4]arene fragments are located in the space, formed by the residues Pro224.A, Gln225.A, Gly449.A, Asp450.A, Ser484.A, Gly509.A, Leu553.A and Pro511.A. The phenol rings of the calixarene cup are in the hydrophobic and stacking interactions with the residues of aromatic aminoacids – Phe482.A, Tyr488.A, and Tyr542.A (Fig. 8).

The second region of thiacalix[4]arene C-1193 binding is in the part of the molecule of Na^+,K^+-ATPase with the segment, bound to the transmembrane spiral M5 and loop L6/7, binding spirals M6 and M7 (L6/7), which can move during the reaction cycle and impact the affinity to the ions, participating in the reaction. It was found that the interaction with the phosphonate groups of calixarene may involve residues Arg28.B and Arg848.A. The linker atoms of sulfur in the calixarene C-1193 "cup" interact with residues Glu847.A and Glu1020.A. Calix[4]arene fragments are located in the space, formed by the residues Tyr40.B, Phe43.B, Tyr44.B, Val298.A, Phe301.A, Leu302.A, Ser851.A, Tyr854.A, Gly855.A and Gln856. A. Here, the calixarene cup is located in the hydrophobic cluster, formed by aromatic aminoacids – Phe33.B, Phe37.B, Tyr40.B, Phe43.B, Tyr44.B, Phe301.A and Tyr854.A. These residues may form hydrophobic and stacking interactions with the phenol rings of the calixarene cup. It should be noted that in region 4, the calixarene molecule forms stacking bonds with the residues of aromatic aminoacids of α- and β-subunit at the same time. In particular, C-1193 interacts with Tyr44.B of the side chain of β-subunit (Fig. 9).

It is obvious that the binding of thiacalix[4]arene C-1193 molecule in the region, close to this segment, may cause disruptions in its conformational mobility, thus impacting the reaction cycle of the enzyme and the affinity to ions, participating in the reaction, and may cause conformational changes in the enzyme structure.

The relevant structural specificities of Na^+,K^+-ATPase may cover the presence of aminoacid residues near the active center, which do not participate directly in the catalysis mechanisms, but spatially close the access to the center of substrate binding. Considering the ability of thiacalix[4]arene C-1193 to penetrate the membrane, its interaction with cytoplasmic fragments of the enzyme is possible.

The intermolecular hydrogen bonds in the "calixarene – protein" system were also investigated. The most notable bond is that of hydrogen, which involves the phosphonate groups and the atoms of calixarene sulfur. The hydrogen bonds with protein atoms are not characteristic of the calixarene cup.

Therefore, the results of the computer simulation, obtained by us, are in agreement with the experimental data about the ability of thiacalix[4]arene to affect the enzymatic activity of Na^+,K^+-ATPase.

Therefore, the results of thiacalix[4]arene C-1193 docking into the binding sites of Na^+,K^+-ATPase demonstrate that this calixarene may be located

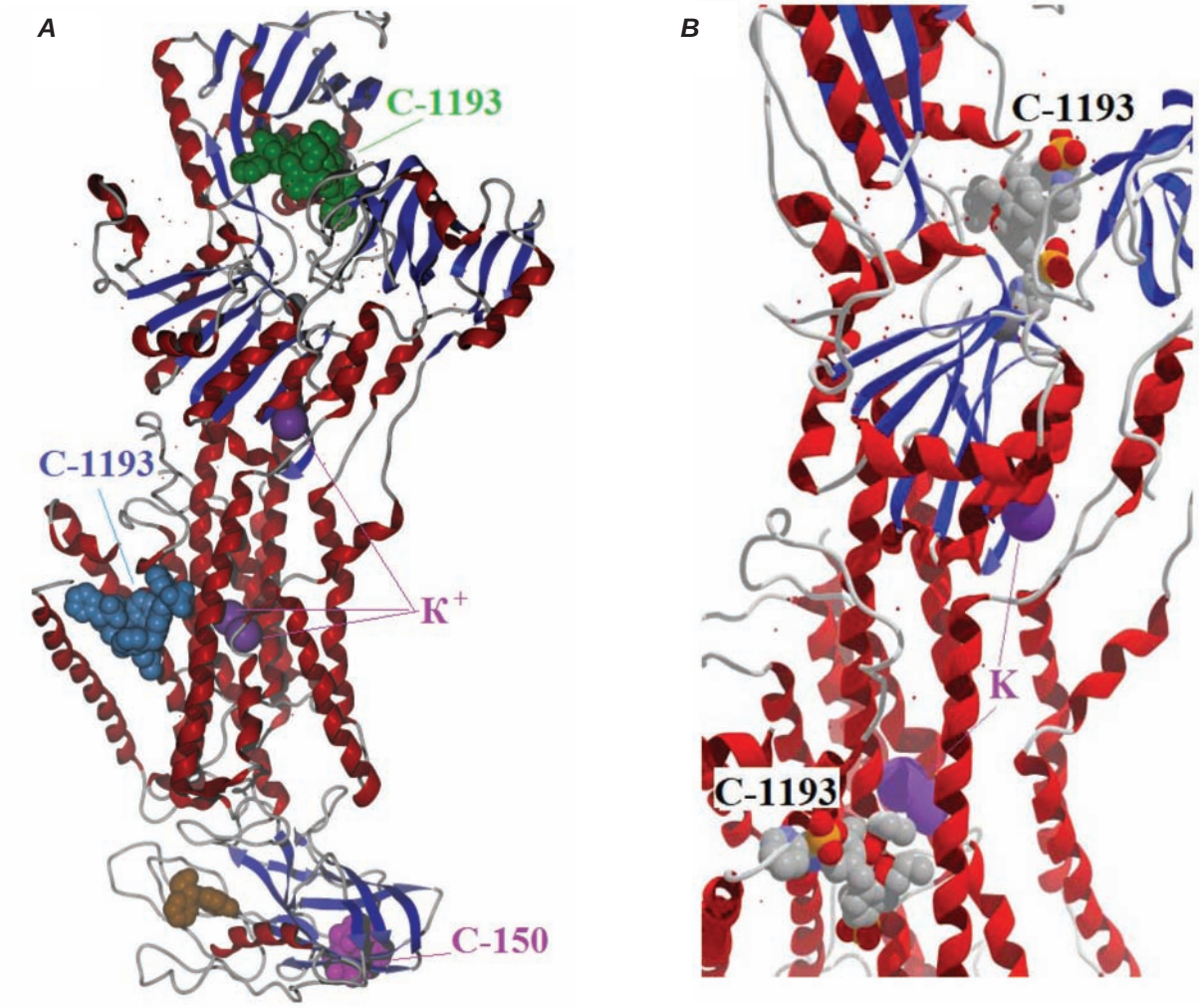

Fig. 7. ***A*** *– The most probable places of C-1193 binding with active sites of Na^+,K^+-ATPase, obtained by the docking method;* ***B*** *– the position of C-1193 after molecular dynamics of the complex of thiacalix[4]arene and N- and transmembrane domains in the interval of 6 ns/1 ns*

in the space of several sites of ligand-binding regions of the enzyme. We determined some specificities in the interaction between the ligand and the enzyme. In particular, a relevant role in the interaction between the calixarene C-1193 cup and the enzyme is played by hydrophobic and steric interactions. It means that the residues of aromatic aminoacids "fix" calixarene in the region of binding to the protein. At the same time, the residues of negatively and positively charged aminoacids form hydrogen bonds with the linker atoms of sulfur in the calixarene "cup" and the oxygen atoms of phosphone fragments of thiacalix[4]arene C-1193. Thus, the hydrophilic fragments of calixarene set "a direction vector" in which the molecule of C-1193 is fixed in the region of interaction with the enzyme.

Considering the complicated structure of this ATPase, which consists of two paired α and β subunits in the biological membrane, C-1193 is likely capable of not only reducing the affinity of Na ions to the sodium pump but also directly affecting the macrostructure of Na^+,K^+-ATPase.

Therefore, the data of this work may serve as a foundation for the elaboration of an effective inhibitor of Na^+,K^+-ATPase of the plasma membrane, based on the investigated thiacalix[4]arene, which, in its turn, will be relevant for further determination of membrane mechanisms of the cation exchange in smooth muscles, for instance, while studying the role of the plasma membrane in ensuring the electromechanical conjugation in them and regulating ion homeostasis in smooth muscle cells.

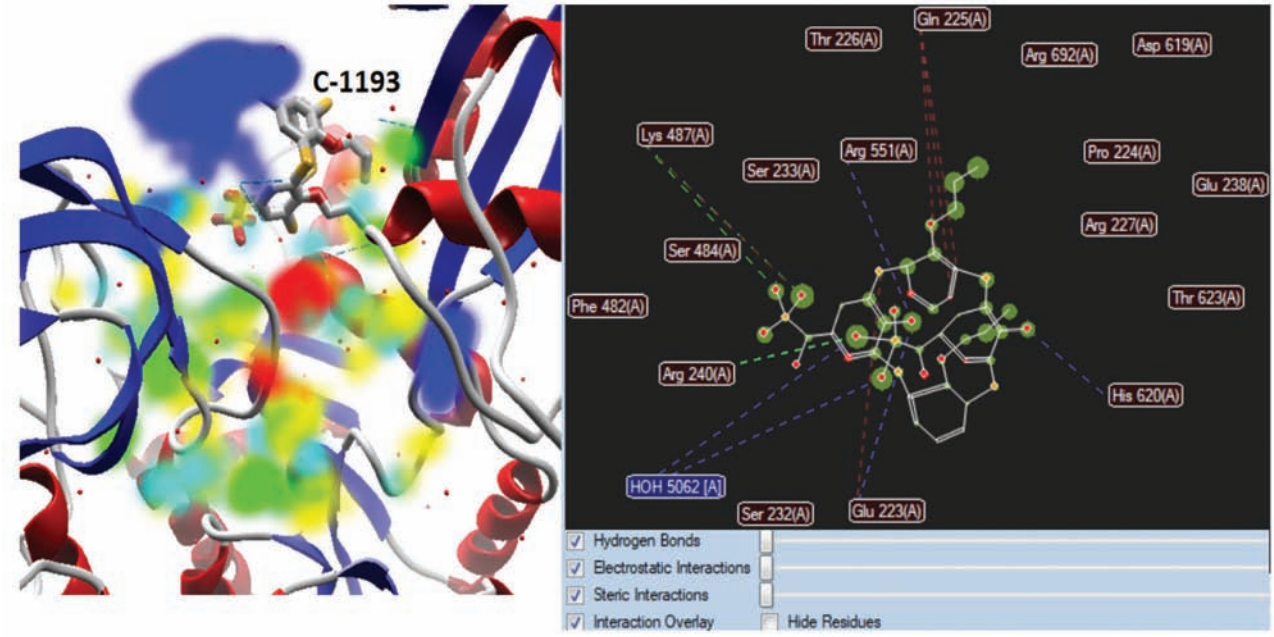

Fig. 8. Different types of interactions between thiacalix[4]arene C-1193 and Na^+,K^+-ATPase in region 1, defined using 4 ns of molecular dynamics. The aminoacid residues, forming H-bonds with the inhibitor, are marked with brown lines. Green color – steric interactions; turquoise color – hydrogen acceptors; yellow – hydrogen donors; red-blue – electrostatic interactions

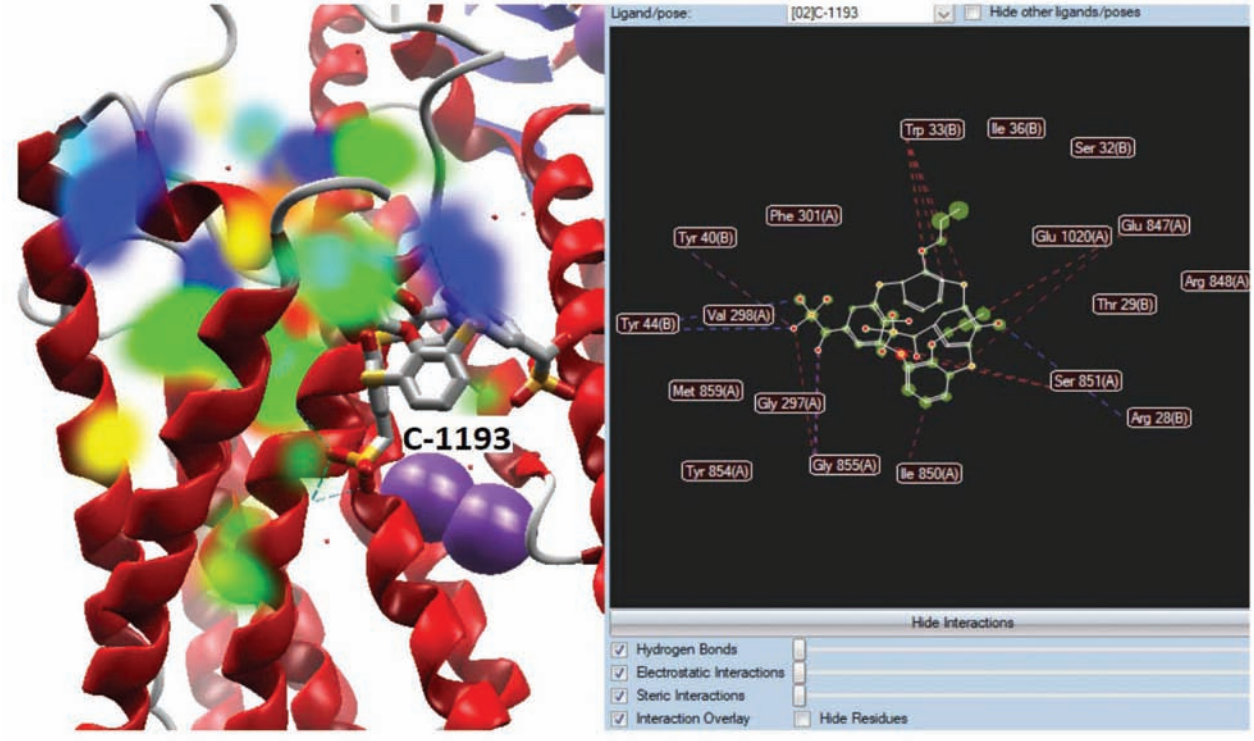

Fig. 9. Different types of interactions between C-1193 and Na^+,K^+-ATPase in region 2, determined by 4 nc of the molecular dynamics. The aminoacid residues, forming H-bonds with the inhibitor, are marked with brown lines. Green color – steric interactions; turquoise color – hydrogen acceptors; yellow – hydrogen donors; red-blue – electrostatic interaction

Conflict of interest. The authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ТІАКАЛІКС[4]АРЕН С-1193 – ПЕРСПЕКТИВНИЙ ІНГІБІТОР НАТРІЄВОЇ ПОМПИ КЛІТИН ГЛАДЕНЬКИХ М'ЯЗІВ МАТКИ

О. В. Малюк[1]✉, Т. О. Векліч[1], О. В. Цимбалюк[2], О. В. Бевза[1], С. О. Черенок[3], А. І. Селіхова[3], В. І. Кальченко[3], С. О. Костерін[1]

[1]Інститут біохімії ім. О. В. Палладіна НАН України, Київ, Україна;
✉e-mail: sanya2000ua@gmail.com;
[2]Навчально-науковий інститут високих технологій, Київський національний університет імені Тараса Шевченка, Київ, Україна;
[3]Інститут органічної хімії НАН України, Київ, Україна

Тіакалікс[4]арен С-1193 (25,27-дибутокси-тіакалікс[4]арен-біс-гідроксиметилфосфонова кислота) виявився високоефективним інгібітором активності Na^+,K^+-АТРази ($I_{0.5}$ = 42,1 ± 0,6 нМ), не впливаючи при цьому на активність Mg^{2+}-АТРази, Ca^{2+}-АТРази та Ca^{2+},Mg^{2+}-АТРази у фракції плазматичної мембрани клітин гладеньких м'язів матки щурів. Досліджено кінетичні закономірності інгібуючої дії С-1193 на активність Na^+,K^+-АТРази. Показано, що С-1193 підвищував константу активації ензиму іонами Na^+, але не K^+. Скоротливу активність матки щурів досліджували тензометричними методами з використанням поздовжніх смужок міометрія з інтактним ендометрієм. С-1193 спричиняв значне підвищення амплітуди ацетилхолін-індукованих скорочень, а також максимальної швидкості фаз скорочення та розслаблення. Водночас впливу С-1193 на скоротливу активність, індуковану селективним агоністом M_3-холінорецепторів цевімеліном, не виявлено. Результати комп'ютерного моделювання показали, що інгібуючий ефект С-1193 пов'язаний з кооперативною дією метилен-бісфосфонатних фрагментів на верхньому краї каліксаренової платформи та атомів сірки-лінкерів «чаші» тіакаліксарену на макроструктуру Na^+,K^+-АТРази.

Ключові слова: тіакалікс[4]арени, Na^+,K^+-АТРаза, плазматична мембрана, клітини гладеньких м'язів, міометрій, комп'ютерне моделювання, докінг.

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