

EXPERIMENTAL WORKS

UDC 577.352.4+544.147+544.176+544.168

doi: <https://doi.org/10.15407/ubj95.05.005>

**A NEW AFFINE INHIBITOR OF SODIUM PUMP
THIACALIX[4]ARENE C-1193 INCREASES
THE INTRACELLULAR CONCENTRATION OF Ca IONS
AND MODIFIES MYOMETRIUM CONTRACTILITY**

T. O. VEKLICH^{1✉}, S. O. CHERENOK², O. V. TSYMBALYUK³, O. A. SHKRABAK¹,
S. O. KARAKHIM¹, A. I. SELIHOVA², V. I. KALCHENKO², S. O. KOSTERIN¹

¹Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine, Kyiv;

✉ e-mail: veklich@biochem.kiev.ua;

²Institute of Organic Chemistry, National Academy of Sciences of Ukraine, Kyiv;

³Educational and Scientific Institute of High Technologies,
Taras Shevchenko National University of Kyiv, Ukraine

Received: 16 June 2023; **Revised:** 31 August 2023; **Accepted:** 27 October 2023

The methods of enzymatic and kinetic analysis were used to demonstrate that thiacalix[4]arene-bis-hydroxymethylphosphonic acid C-1193 had the inhibitory effect ($I_{0.5} = 42.1 \pm 0.6$ nM) on Na^+, K^+ -ATPase activity in the plasma membrane of myometrium cells with no effect on the relative activity of other ATPases localized in this subcellular structure. The method of confocal microscopy and Ca^{2+} -sensitive fluorescent probe fluo-4 were used to demonstrate that thiacalix[4]arene C-1193 increased the intracellular concentration of Ca ions in the immobilized uterine myocytes. The tenzometric studies proved that C-1193 (10 and 100 μM) increased the isometric phasic contractions, induced via the paths of both electromechanical (depolarization with high-potassium solution) and pharmacomechanical (application of uterotonic hormone oxytocin, neurotransmitter acetylcholine or selective agonist of muscarinic acetylcholine receptors cevimeline) coupling. Application of thiacalix[4]arene C-1193 as a selective and effective inhibitor of Na^+, K^+ -ATPase may be useful both for studying the regulation of ion homeostasis in smooth muscle cells and creation of new uterotonics based on the calixarene core.

Key words: Na^+, K^+ -ATPase, myometrium, thiacalix[4]arene, contractility mechanokinetics.

Nowadays the search for novel low-molecular regulators of membrane-bound ion transport systems, including active transport, is required for further studies on biochemical mechanisms of electro- and pharmacomechanical coupling in muscles, including smooth muscles (SM). In addition, the findings of this search could have many practical prospects, as selective highly-affine reverse effectors, capable of modifying the activity of specific ion-transporting systems, can serve as a basis for the elaboration of new-generation pharmacological preparations to correct the contractile function of SM, if it is impaired due to pathological states. Among SM organs, the uterus has an exclusive role, explained by its unique function in the organism –

child-bearing and delivery. The impaired contractile activity of myometrium is the foundation for various pathologies: slow labor, spontaneous abortions, early labor, miscarriages, atony, hypo- and hypertonicity of the uterus [1-3]. Thus, there is an urgent need to elaborate novel efficient medications capable of preventing and counteracting the impairments in myometrium functioning.

A number of cation-dependent ATPases are present in the plasma membrane (PM) of smooth muscle cells. For instance, fundamental significance is attributed to electroenzyme Na^+, K^+ -ATPase, supporting high K^+ concentrations and low Na^+ concentrations in the cytoplasm, ensuring the excitability of the cell and other biochemical and biophysical pro-

cesses. This enzyme is present in all the excitable tissues, and its activity is very sensitive to the energy state of the cell; thus, to some extent, it may be assumed that the catalytic and transporting activity of Na^+, K^+ -ATPase characterizes the energy potential of the cell. Na^+, K^+ -ATPase has a key role in the regulation of many biochemical processes; it is a target for both physiological regulatory factors and pharmacological agents [4-8].

The targeted synthesis and the study of the properties of effectors capable of penetrating via PM and changing the enzymatic or transporting activity of some intracellular membrane-bound and cytoplasmic proteins in a reversible way and with high affinity and selectivity is one of the most important tasks of modern biological, biophysical, and bioorganic chemistry. Indeed, the elaboration of novel, highly effective low-toxic selective inhibitors and activators of enzymes is extremely necessary, in particular, for the purpose of further research on ion, molecular, and membrane mechanisms of intracellular signaling. In this context, one should consider calixarenes, "cup"-like macrocyclic compounds, whose specifics of three-dimensional molecular structure and practically unlimited synthetic potential allow for rational designing of targeted effectors, to be rather promising agents [9-11].

The unique structure and properties of calixarene molecules allow for the modification of their structure (involving different functional groups on the upper and lower rims of the molecule); thus, the search for new calixarene effectors of enzymes has only begun. It should be noted that calixarenes are remarkable for their low toxicity [12] and immunogenicity [9, 11, 12], as well as affordable and low-cost synthesis.

In our previous experiments, we found that some calix[4]arenes can be selective inhibitors of Mg^{2+} -dependent ATP-hydrolases, which are the systems of energy-dependent transportation of cations, including $\text{Na}^+, \text{K}^+, \text{Mg}^{2+}$ -dependent ATP-hydrolase (Na^+, K^+ -ATPase, sodium pump) and $\text{Ca}^{2+}, \text{Mg}^{2+}$ -dependent ATP-hydrolase (calcium pump) of the plasma membrane [13-15].

This article presents new findings obtained by us while studying the regularities in the effect of thiacalix[4]arene C-1193, the inhibitor of Na^+, K^+ -dependent ATP-hydrolase of the plasma membrane, on the intracellular concentration of Ca ions in uterine myocytes and the mechanokinetics of contractility. In the enzymological part of this study, we compare

the effects of the inhibitory activity of a novel thiacalix[4]arene C-1193 with a bigger size, containing four sulfur atoms in the macrocyclic platform, and the effects of calix[4]arene C-99, the impact of which on Na^+, K^+ -ATPase was studied by us before [16, 17].

Materials and Methods

A. Synthesis of (thia)calix[4]arene-bis-hydroxymethylphosphonic acids C-99 and C-1193. The NMR spectra were registered on a Varian VXR-400 spectrometer operating at 399.987 MHz (^1H), 1050.8 MHz (^{13}C), using TMS as a reference. The ^{31}P NMR spectra were recorded on a Varian VXR-400 spectrometer operating at 162 MHz using 85% H_3PO_4 as reference. The melting points were measured on a Boëtius heating block and not corrected. The reactions were carried out in anhydrous solvents.

5,17-diformyl-25,27-dipropoxycalix[4]arene **1a** [18], 5,17-diformyl-25,27-dibutoxythiacalix[4]arene [19] and tris-trimethylsilylphosphite [20] were synthesized according to the literature methods.

The general method of synthesizing C-99 and C-1193. Tris-trimethylsilylphosphite (5 mmol) was added to a solution of diformylthiacalixarene **1** (1 mmol) in dry methylene chloride (20 ml) at room temperature. The mixture was stirred for 15 h. Methylene chloride and silyl phosphite residues were evaporated under reduced pressure and kept in a vacuum of 0.1 mm Hg at 50°C for 2 h. Wet methanol (30 ml) was added to the obtained silyl derivative of thiacalixarene **2** and the reaction mixture was stirred for 2 h at 40°C. The solvent was evaporated under reduced pressure; the residue was kept in a vacuum of 0.1 mm Hg at 50°C for 2 h. C-99 and C-1193 acids were dissolved in methanol (10 ml) and diluted with water (10 ml). The precipitate formed was filtered, dried in a vacuum of 0.1 mm Hg at room temperature for 5 h.

25,27-dipropoxycalix[4]arene-bis-hydroxymethylphosphonic acid C-99. Colorless crystals: yield 90%. m.p. 263-266°C. (melt.temp. 262-266°C [21]). ^1H NMR (DMSO), δ : 1.30 (t, 6H, J 7.5 Hz, CH_2CH_3), 1.99 (m, 4H, $\text{CH}_2\text{CH}_2\text{CH}_3$), 3.37 (d, 4H, J 13.0 Hz, $\text{ArCH}_{2\text{eq}}$), 3.94 (t, 4H, J 6.2 Hz, OCH_2), 4.24 (d, 4H, J 13.0 Hz, $\text{ArCH}_{2\text{ax}}$), 4.50 (d, 2H, J 10.0 Hz, PCH), 6.75, (t, 2H, J 8.0 Hz, p-ArH), 7.11, 7.14 (two d, 4H, J 8.0 Hz, m-ArH), 7.24 (s, 4H, m-ArH). ^{31}P NMR (DMSO), δ 18.45. ^{13}C NMR (DMSO), δ 10.1, 20.35, 31.01, 70.6 (d, JPC = 159 Hz), 78.47, 125.5, 127.5, 128.1, 129.2, 131.1, 134.2, 151.7, 151.78, 152.17; Mass

(FAB) m/z ; 729 $[M + H]^+$, 752 $[M + Na]^+$, 1458 $[2M + H]^+$. Calcd. for $C_{36}H_{42}O_{12}P_2$ 728.66.

26,28-dihydroxy-25,27-dibutoxythiacalix[4]arene-5,17-bis(α -hydroxymethylphosphonic acid) C-1193. Colorless crystals: yield 92 %, m.p. 221–224°C. 1H NMR (DMSO), δ : 1.03 (t, 6H, J 7.5 Hz, CH_2CH_3), 1.58 (m, 4H, $CH_2CH_2CH_3$), 1.88 (m, 4H, $CH_2CH_2CH_3$), 4.4 (t, 4H, J 6.2 Hz, OCH_2), 4.68 (d, 2H, J 12.5 Hz, PCH), 6.66 (t, 2H, J 8.0 Hz, p-ArH), 6.94, 7.00 (two d, 4H, J 8.0 Hz, m-ArH), 7.68 (s, 2H, OH), 7.70, 7.75 (two s, 2H+2H, m-ArH). ^{31}P NMR (DMSO), δ 18.1. ^{13}C NMR (DMSO), δ 14.24, 19.07, 32.06, 69.6 (d, JPC = 159 Hz), 75.56, 121.27, 121.52, 125.91, 129.34, 132.28, 135.81, 136.16, 156.63, 158.39; Mass (FAB) m/z ; 840 $[M + H]^+$. Calcd. for $C_{34}H_{49}O_{12}P_2S_4$ 839.16.

B. Biochemical studies.

Preparative biochemistry. The PM fraction of uterine SM cells was isolated from the porcine myometrium as described before [22].

The protein content in the membrane fraction was determined by the method of M. Bradford [23] using the reaction with Coomassie reagent – G250.

The myocytes were isolated from the uterus of non-pregnant laboratory rats using collagenase and soy inhibitor trypsin by Mollard's method [24].

The work was conducted in accordance with the Declaration of Helsinki (World Medical Assembly, 1964), the International principles of the European Convention for the Protection of Vertebrate Animals, Used for Experimental and Other Scientific Purposes (Strasbourg, 1986), the Declaration of Principles on Tolerance (28th UNESCO Assembly, 1995), the Universal Declaration on Bioethics and Human Rights (UNO, 1997), the norms of the Convention for the Protection of Human Rights, adopted in 1997 due to the introduction of new biomedical technologies, in Oviedo (Spain) and ratified by the Verkhovna Rada of Ukraine in 2002, the Law of Ukraine No. 3447 IV "On Protection of Animals from Cruelty".

Enzymological studies. The total ATPase activity was determined in the PM fraction of myometrium cells, as described before [21], at 37°C in the standard medium (volume – 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 presence of Ca^{2+} -chelating agent of EDTA in the incubation medium ensured the binding of endogenous ions of Ca therein. The amount of membrane fraction protein in the

probe was 20–30 μg . The incubation time at 37°C was 4 min. The enzymatic reaction was initiated by the introduction of the aliquot (50 μl) of PM fragment suspension (8°C) to the incubation medium, and terminated by the introduction of 1 ml of "stop"-solution to the incubation mixture as follows: 1.5 M sodium acetate, 3.7% formaldehyde, 14% ethanol, 5% TCA, pH 4.3 (at 8°C).

Na^+, K^+ -ATPase activity was estimated by the difference between the values of the total ATPase activity and the basal Mg^{2+} -ATPase activity. The basal Mg^{2+} -ATPase activity was determined in the same medium but in the presence of 1 mM ouabain (selective inhibitor of Na^+, K^+ -ATPases of PM [25, 26]).

The incubation medium, with a composition, similar to the above described one but lacking fragments of plasma membranes, served as a control for non-enzymatic hydrolysis of ATP. The control of the amount of endogenous non-organic phosphorus (P_i) in the membrane preparation was the medium, containing only the suspension of the membrane preparation in the aqueous solution. The basal ATPase enzymatic activity was estimated as a difference between the amount of P_i , which was formed in the incubation medium in the presence and in the absence of PM fragments with the consideration of the amendment for non-enzymatic hydrolysis of ATP and the content of endogenous P_i in the membrane preparation. The amount of P_i reaction product was determined by the method [27].

In our experiments, the average value of relative activities of Na^+, K^+ -ATPase and basal Mg^{2+} -ATPase of PM was 10.2 ± 0.7 and 18.1 ± 1.2 $\mu mol P_i/mg$ of protein per one hour, respectively ($n = 7$).

Ca^{2+}, Mg^{2+} -ATPase activity was estimated by the difference between the values of ATPase activities in the presence and in the absence of exogenous Ca^{2+} (against the background of 1 mM EDTA – specific chelating agent for Ca^{2+}) in the incubation medium. In the sarcolemma of the porcine myometrium, the relative enzymatic activity of Ca^{2+}, Mg^{2+} -ATPase was 3.4 ± 0.3 $\mu mol P_i/mg$ of protein per one hour, respectively ($n = 7$).

It should be noted that the PM of uterine myocytes was found also to contain Ca^{2+} -ATPase, the properties of which differ from Ca^{2+}, Mg^{2+} -ATPase, since its activity was manifested in the presence of millimolar concentrations of Ca^{2+} and ATP in the incubation medium against the background of Mg^{2+} [28, 29]. Ca^{2+} -ATPase was of low affinity to the activating cation – the constant of activation

with Ca^{2+} for K_{Ca} was 1 mM [29]. The low-affinity Mg^{2+} -independent Ca^{2+} -ATPase activity was determined in the PM fraction of myometrium cells at 37°C in the medium (volume – 0.4 ml), containing (mM): 1 ATP, 3 CaCl_2 , 125 NaCl, 25 KCl, 1 EDTA, 20 Hepes-tris-buffer (pH 7.4), 1 NaN_3 , 1 ouabain, 0.1 μM thapsigargin, and 0.1 % digitonin. The mentioned Ca^{2+} -ATPase activity was estimated as a difference between the amount of P_i , which was formed in the incubation medium in the presence and in the absence of PM fraction with the consideration of the amendment for the content of endogenous P_i in the membrane preparation. In the sarcolemma of the porcine myometrium, the relative enzymatic activity of low-affinity Mg^{2+} -independent Ca^{2+} -ATPase of PM was $12.7 \pm 2.0 \mu\text{mol P}_i/\text{mg}$ of protein per hour, respectively ($n = 7$).

In the experiments on the impact of different concentrations of calixarene compounds C-99 and C-1193 (1–100 μM) on ATP-hydrolase activity, the above-described standard incubation medium was used with the addition of the aliquot of the solution of thiacalix[4]arene in the relevant concentration. The experiments involved the use of the concentrated (20 mM) solutions of C-99 and C-1193 in DMSO, which were further diluted with water.

Determining the changes in the concentration of intracellular Ca^{2+} with the addition of confocal microscopy. To register the changes in the concentration of Ca^{2+} in SM cells, the suspension of myocytes, obtained by [24], was loaded with Ca^{2+} -sensitive probe fluo-4 AM at room temperature for 20 min, then the cells were precipitated by centrifugation for 15 min at 1,000 g, diluted in the isotonic storage medium (containing 25 mM HEPES-KOH (pH = 7.4; 8°C, 150 mM NaCl) and applied to a glass surface with poly-L-lysine. The obtained preparation of the immobilized cells was investigated with the laser scanning confocal microscope LSM 510 META. For analysis, we selected the spindle-shaped cells with a clearly defined nucleus, stained with DNA-sensitive fluorescent probe Hoechst, (applied 10 min before the registration). To register the relative changes in the concentration of Ca^{2+} in the cytoplasm, a series of consecutive photographs were taken, during which the aliquot of the C-1193 solution in the concentration of 20 μM (5 μl) was added.

Kinetic estimates. To study the concentration dependence of the effect of calix[4]arenes on the enzymatic activity, the values of inhibition coefficients $I_{0.5}$ and Hill coefficients n_H were estimated using the

linearized charts of Hill according to the equation $\lg[(A_{\text{max}} - A)/A] = -n_H \lg I_{0.5} + n_H \lg [C-1193]$, where A_{max} and A – relative enzymatic activities in the absence (“zero point”) and in the presence of calix[4]arene in the incubation medium in the concentration [C-1193].

Tenzometric experiments. The contractile activity in the preparations of longitudinal SM of uterine horns with preserved endothelium was registered in the isometric mode. Muscle stripes (2×10 mm) were placed into the working chamber (the volume of 2 ml) with the flowing Krebs solution (the flow rate of 5 ml/min), thermostated at 37°C. The preparations were provided with passive tension at the rate of 10 mN and left for 1 h until achieving stable reproduction of contractions. The signals were registered with an analogue-to-digital transformer.

The Krebs solution was used in the experiments (mM): 120.4 NaCl; 5.9 KCl; 15.5 NaHCO_3 ; 1.2 NaH_2PO_4 ; 1.2 MgCl_2 ; 2.5 CaCl_2 ; 11.5 glucose; pH of the solution was 7.4. The high-potassium solution (HPS), containing K^+ in the concentration of 80 mM, was prepared by isotonic replacement of the required amount of Na^+ in the initial Krebs with the equimolar amount of K^+ . Also, the study involved the use of acetylcholine (10 μM), cevimeline (100 μM), and oxytocin (0.1 IU).

Thiacalix[4]arene C-1193 was applied in concentrations of 10 and 100 μM ; it was preliminary dissolved in DMSO and added to the working solution to obtain the final aliquot of this organic solvent of 0.1% from the total volume of the solution. The control contractions were registered against the background of 0.1% DMSO.

Mechanokinetic analysis of contractions. The study of the spontaneous contractile activity in SM preparations was conducted according to the empirical multiparameter method of the complex mechanokinetic analysis, previously developed by us [30]. To analyze the complete profile of single spontaneous contractions, they were linearized in the coordinates $[\ln(f_R/f_C) \text{ vs } \ln(1+\Delta t/t)]$, where f and t – instant values of force and time at the level of the contraction cycle (C and R – symbols for the phases of contraction and relaxation, respectively), F_C and F_R – the values of the force at the inflexion points of the mechanogram at the level of the phases of contraction (from the beginning of the increase in the force to its maximal value F_{max}) and relaxation (from the maximal value of the force F_{max} at the time moment τ_0 and until its return to the basal level), Δt – arbitrary fixed time interval (which varied within 15–50 s). The lineariza-

tion charts were used to determine the characteristic constants k and n , which were further used to calculate the parameters: time (τ_0 , τ_C and τ_R), force ($F_{\max}$, F_C and F_R), velocity (V_C and V_R), and impulse (I_0 , I_C and I_R) parameters. Here, V_C and V_R – maximal velocities of the phases of contraction and relaxation, respectively, I_0 , I_C and I_R – force impulses at the level of amplitude and maximal velocities of contraction and relaxation, respectively. Spontaneous contraction, registered within 10–30 min from the start of C-1193 application were used in the analysis.

The analysis of kinetic properties of the induced contractions was made according to the method [31]. The analysis involved the estimation of the indices, independent from the amplitude of contractile responses, the maximal velocities of the contraction phase (V_{nc}) and relaxation phase (V_{nr}), normalized in terms of the amplitude. The induced contractions registered within 20–30 min from the start of C-1193 application were used in the analysis.

Statistical analysis. The statistical analysis of the data obtained was conducted by the methods of variation statistics. Data were normally distributed. The Student's t -test was used to determine the reliable differences between the mean values of samplings. In all cases, the results were considered reliable on condition of $P < 0.05$. The validation analysis of data approximation by the linear function was performed using Fisher's F -test; the value of the determination coefficient (R^2) was at least 0.96 in all cases. The results were presented as the mean $\pm$ standard error of mean value, n – number of experiments. The kinetic and statistical calculations were done using the programs MS Excel and Origin 2018.

Reagents. The following reagents were used in the experiments: ATP, Hepes, ouabain, thapsigargin, Hoechst, fluo-4 AM, collagenase, poly-L-lysine, acetylcholine, cevimeline (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

(Thia)calixarenes C-99, C-1193 were obtained by the one-pot synthesis, envisaging two chemical stages (Scheme). In the first stage, the interaction between diformyl(thia)calixarenes **1a,b** and tris-trimethylsilylphosphite was used to obtain silyl derivatives of (thia)calixarene-bis-hydroxymethylphospho-

nic acids **2a,b**. During the processing of the obtained silyl derivatives **2a,b** with methanol, the bonds of P-O-Si and C-O-Si get bifurcated, and the target (thia)calixarene-hydroxymethylphosphonic acids C-99, C-1193 are formed. The yields of reaction products in both stages are practically quantitative.

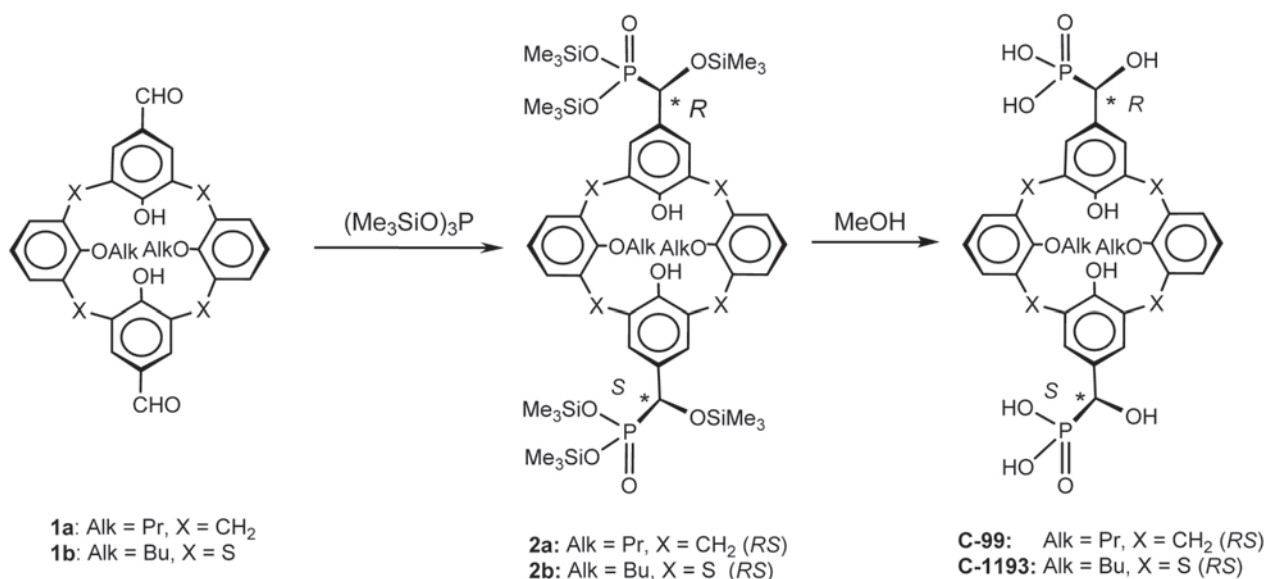
The binding of tris-trimethylsilylphosphite to C=O bonds of formyl groups of calixarenes **1a,b** occurs in a diastereoselective way with the formation of only *mezo*-form of silyl derivatives **2a,b** with R and S configuration of chiral carbon atoms of phosphonomethylol fragments of CH(OH)P. This is evident in the same set of signals in ^1H , ^{13}C and ^{31}P NMR spectra of the acids C-99 and C-1193 (SI5 – SI10), obtained on their basis. Here, the spectra and physical-chemical properties of acid C-99 coincide with the corresponding characteristics of the *mezo*-form of this compound, previously obtained by diastereoselective binding of dialkyl esters of phosphorous acid to diformylcalixarene **1a** [21].

The obtained acids C-99, C-1193 have a cone-shaped conformation of the macrocyclic frame. As for acid C-99, the cone-shaped conformation is clearly confirmed by the presence of two duplets of the spin system AB of axial and equatorial protons of ArCH_2Ar groups with chemical shifts of 3.37 and 4.24 ppm respectively, and the constants of spin-spin interaction $^2J_{\text{HH}}$ 13.0 Hz. As for thiacalixarene-bis-hydroxymethylphosphonic acid C-1193, the cone-shaped conformation is demonstrated in the values of chemical shifts of the protons in OCH_2 groups at 4.40 ppm. In the case of conformation 1,3-alternate, the signals of these groups should be considerably shifted to the strong field of δ 3.81–3.86 ppm due to the occurrence of OCH_2 groups into the cones of screening for two *sin*-oriented phenyl rings of the macrocyclic frame [32, 33].

The cone-shaped conformation of C-99 and C-1193 is also confirmed by the data of molecular modelling using HyperChem program (Fig. 1).

In the obtained models, the phosphorylated benzene rings have coplanar orientation and para-substituted benzene rings – perpendicular orientation regarding the main plane of the macrocycle, formed by linker atoms of carbon or sulfur.

Here, the geometrical parameters of energetically minimized structures of C-99, C-1193, namely, the distances between phosphorus atoms (5.09 Å and 4.14 Å, respectively), the distances between diametrical linker atoms of carbon (7.21 Å) and sulfur (8.04 Å) and dihedral angles, formed by benzene



Scheme. Diastereoselective synthesis of (thia)calixarene-hydroxymethylphosphonic acids C-99, C-1193

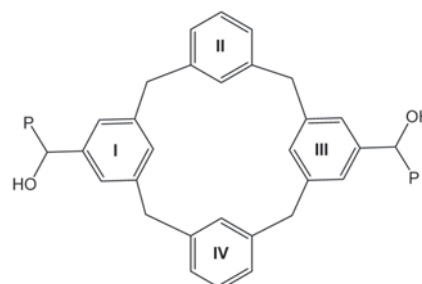
rings with the main plane of the macrocycle (Table), are different.

Therefore, the replacement of methylene linkers in the macrocyclic platform C-99 with sulfur atoms (molecule C-1193) changes the geometric parameters of the macrocycle and impacts the biological activity of the molecule.

In our previous studies, we showed that calixarene C-99 in the concentration of 100 μ M inhibited the activity of Na⁺,K⁺-ATPase in PM of uterine myocytes as compared to the control [16]. At the same time, it practically did not impact the basal Mg²⁺-ATPase activity (the decrease in the activity was only down to 91% as compared to the control value) (Fig. 2).

Therefore, in our further experiments, we studied the effect of a number of calix[4]arenes, struc-

Table. The dihedral angles ($^{\circ}$), formed by benzene rings I, II, III, IV with the main plane of the macrocycle in calix[4]arene C-99 and thiacalix[4]arene C-1193



Structure	I	II	III	IV
C-99	105	124	105	118
C-1193	108	132	64	135

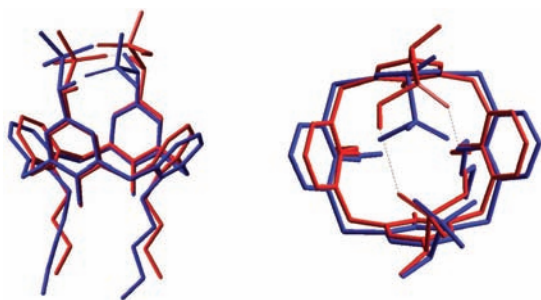


Fig. 1. Energetically minimized structures of calix[4]arene C-99 (in red) and thiacalix[4]arene C-1193 (in blue) (HyperChem)

tural analogues of calix[4]arene C-99, on Na⁺,K⁺-ATPase activity of PM [17], and found that thiacalix[4]arene C-1193 had an even higher inhibitory effect.

We tried to determine the selectivity of the effect of thiacalix[4]arene C-1193 on ATP-hydrolase activities of PM in myocytes and demonstrated that thiacalix[4]arene C-1193 in the concentration of 100 μ M effectively inhibited Na⁺,K⁺-ATPase activity of PM in myometrium cells to the level of $5.2 \pm 0.8\%$ as compared to the control value (accepted as 100%) ($n = 5$) (Fig. 2).

At the same time, this substance, used in the same concentration, practically did not impact the enzymatic properties of basal Mg^{2+} -ATPase, Ca^{2+} -ATPase and $\text{Ca}^{2+},\text{Mg}^{2+}$ -ATPase of PM: the corresponding activities were 97.1 ± 0.8 ; 95.2 ± 0.9 and $93.1 \pm 1.1\%$ regarding the control value ($n = 5$) (Fig. 2). Therefore, thiacalix[4]arene C-1193, used in the concentration of $100 \mu\text{M}$, effectively inhibited the activity of Na^+, K^+ -ATPase in the PM fraction of uterine myocytes. At the same time, it almost did not impact the activity of other ATP-hydrolases in PM. Thus, thiacalix[4]arene C-1193 selectively (at the PM level) inhibits the activity of Na^+, K^+ -ATPase of PM, not affecting the activities of Mg^{2+} -ATPase, Ca^{2+} -ATPase and $\text{Ca}^{2+},\text{Mg}^{2+}$ -ATPase of PM.

In our further studies, we investigated the concentration dependence of the inhibitory effect of calix[4]arene C-99 and thiacalix[4]arene C-1193 (10^{-8} – 10^{-4} M) on the activity of Na^+, K^+ -ATPase of PM. As seen in Fig. 3, both compounds inhibit Na^+, K^+ -ATPase activity of PM in a dose-dependent way. However, thiacalix[4]arene C-1193 acts with higher efficiency than calix[4]arene C-99. The estimated values of the inhibition coefficient $I_{0.5}$ are 98 ± 8 nM and 42.1 ± 0.6 nM, the values of Hill coefficient n_H are 0.27 ± 0.03 and 0.36 ± 0.05 for calix[4]arene C-99 and thiacalix[4]arene C-1193, respectively ($n = 5$).

Thus, the experiment results demonstrate that the investigated thiacalix[4]arene C-1193 highly

effectively ($I_{0.5} = 42.1 \pm 0.6$ nM) inhibits the enzymatic activity of Na^+, K^+ -ATPase of PM, not affecting the activities of Mg^{2+} -ATPase, Ca^{2+} -ATPase and $\text{Ca}^{2+},\text{Mg}^{2+}$ -ATPase of PM (Fig. 2, 3).

It is known that PM of uterine myocytes has a functioning energy-dependent system of $\text{Na}^+/\text{Ca}^{2+}$ -exchange, this antiport cation exchange is ensured with the energy of transmembrane sodium gradient, created due to the functioning of the sodium pump [34–36]. Since thiacalix[4]arene C-1193 inhibited the activity of Na^+, K^+ -ATPase of plasma membrane effectively, it should have been expected that the mentioned inhibitory effect would be accompanied with the increase in the concentration of Ca^{2+} ions in the myoplasm due to the inhibition of Na^+ -dependent release of Ca^{2+} from myocytes. Thus, in our further experiments, we tried to find out whether thiacalix[4]arene C-1193 would affect the intracellular concentration of Ca^{2+} in smooth muscle cells. In these experiments, we estimated the changes in the concentration of Ca^{2+} in myocytes under the effect of thiacalix[4]arene C-1193, using the method of confocal microscopy and Ca^{2+} -sensitive probe fluo-4. It was demonstrated that under the effect of thiacalix[4]arene C-1193 ($20 \mu\text{M}$), there was a sharp increase in the fluorescent response of Ca^{2+} -sensitive probe fluo-4 AM in the cell (Fig. 4 and Fig. 5). Within the next two minutes, the concentration of Ca^{2+} decreased which demonstrated the involvement of other ener-

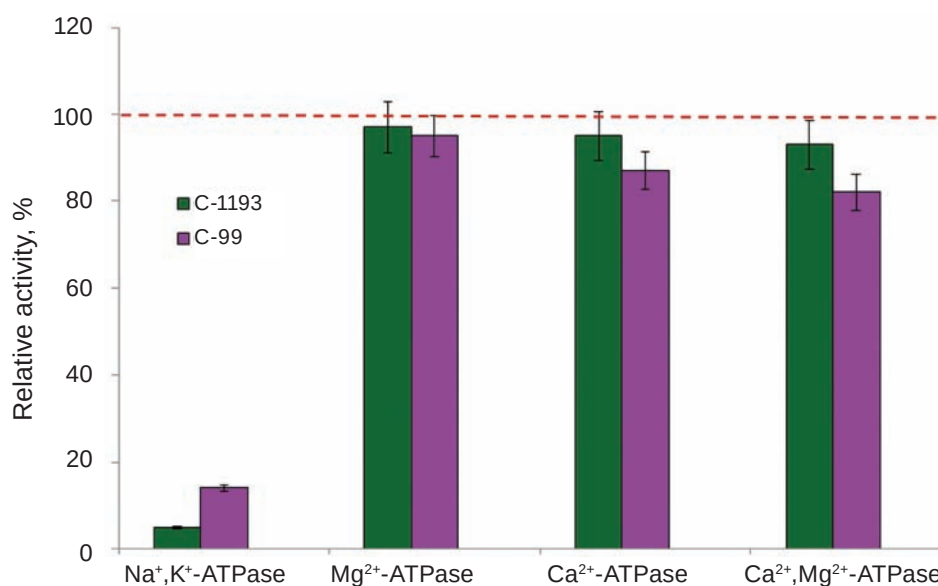


Fig. 2. The results of the study on the comparative effect of calix[4]arene C-99 and thiacalix[4]arene C-1193 ($100 \mu\text{M}$) on ATP-hydrolase activities of plasma membranes of the myometrium cells ($n = 5$). The values of enzymatic activities in the absence of calix[4]arenes in the incubation medium are accepted as 100%

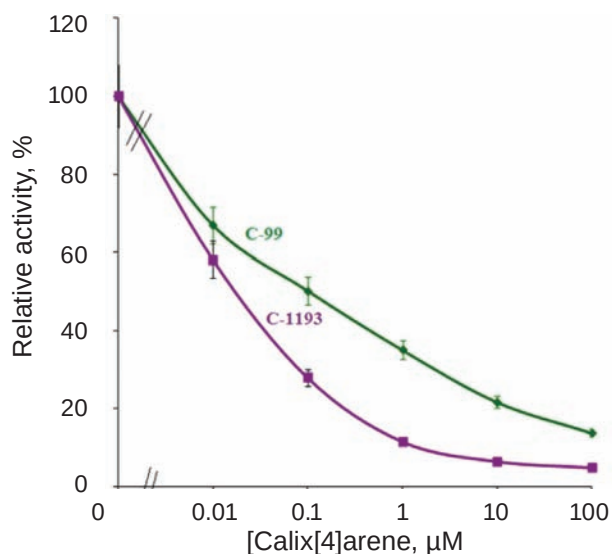


Fig. 3. The concentration dependencies of the effect of calix[4]arene C-99 and thiacalix[4]arene C-1193 on the activity of Na^+, K^+ -ATPases in plasma membranes of the myometrium cells ($n = 5$). The values of specific enzymatic activity in the absence of calix[4]arenes in the incubation medium are accepted as 100%

gy-dependent mechanisms, regulating the homeostasis of Ca^{2+} concentration ($\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPases of PM and sarcoplasmic reticulum, Ca^{2+} -uniporter of mitochondria). There remained a stable fluorescence level of the DNA-sensitive probe, Hoechst, which was mainly localized in the nucleus of smooth muscle cells. So thiacalix[4]arene C-1193 is a selective inhibitor of Na^+, K^+ -ATPase of PM increased cytosolic Ca^{2+} concentration in smooth muscle cells.

The previous series of experiments clearly demonstrated that thiacalix[4]arene C-1193 inhibited Na^+, K^+ -ATPases in PM and induced considerable changes in the homeostasis of Ca^{2+} ions in myocytes, so one could assume that it was also capable of changing the functional properties of the integral SM tissue. So, our subsequent studies were related to the investigation of spontaneous and induced contractile activity of pluricellular smooth muscle preparations of uterine horns of non-pregnant rats.

Thiacalix[4]arene C-1193 induced dose-dependent inhibition of spontaneous contractions in the myometrium. For instance, under the effect of this compound in the concentration of 100 μM ,

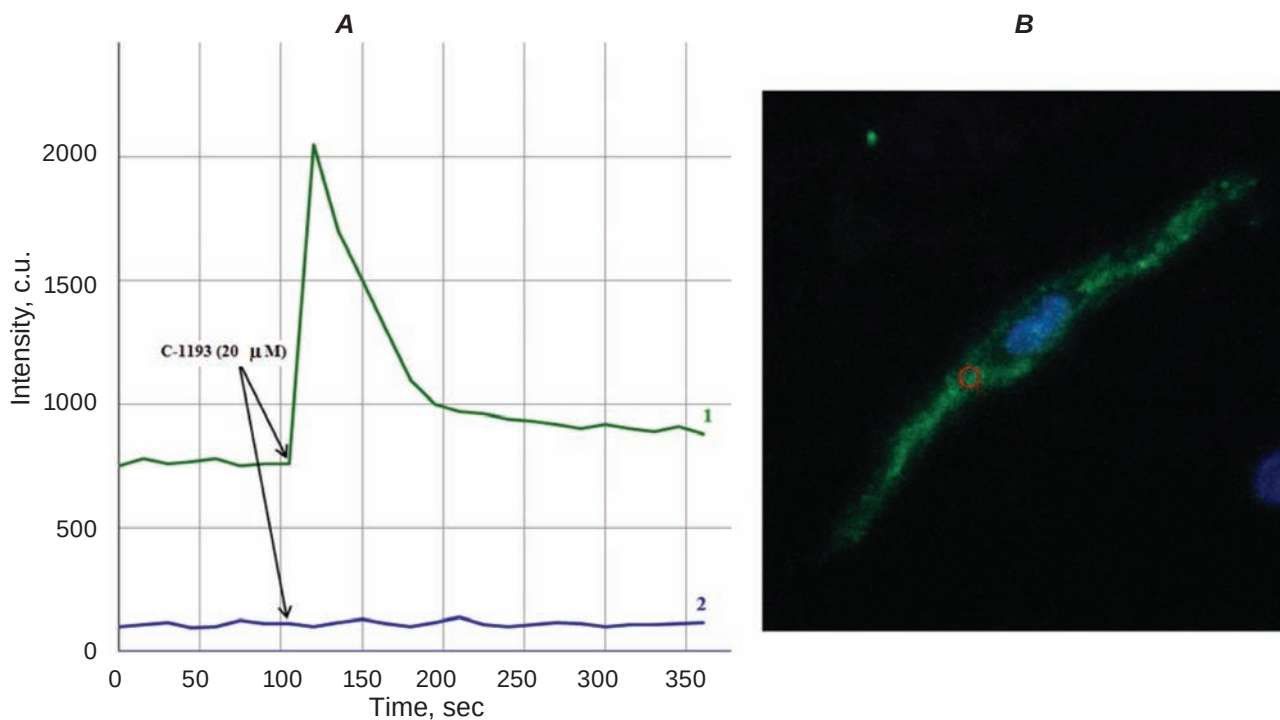


Fig. 4. **A.** The fluorescence intensity of probes of Ca^{2+} -sensitive fluo-4 AM (1) and DNA-sensitive Hoechst (2) in SM under the effect of thiacalix[4]arene C-1193. **B.** The image of a smooth muscle cell in the uterus, obtained using the scanning laser confocal microscope. The introduction of the aliquot of the thiacalix[4]arene C-1193 solution (the final concentration – 20 μM) is indicated with the asterisks. The results of the typical experiment are presented

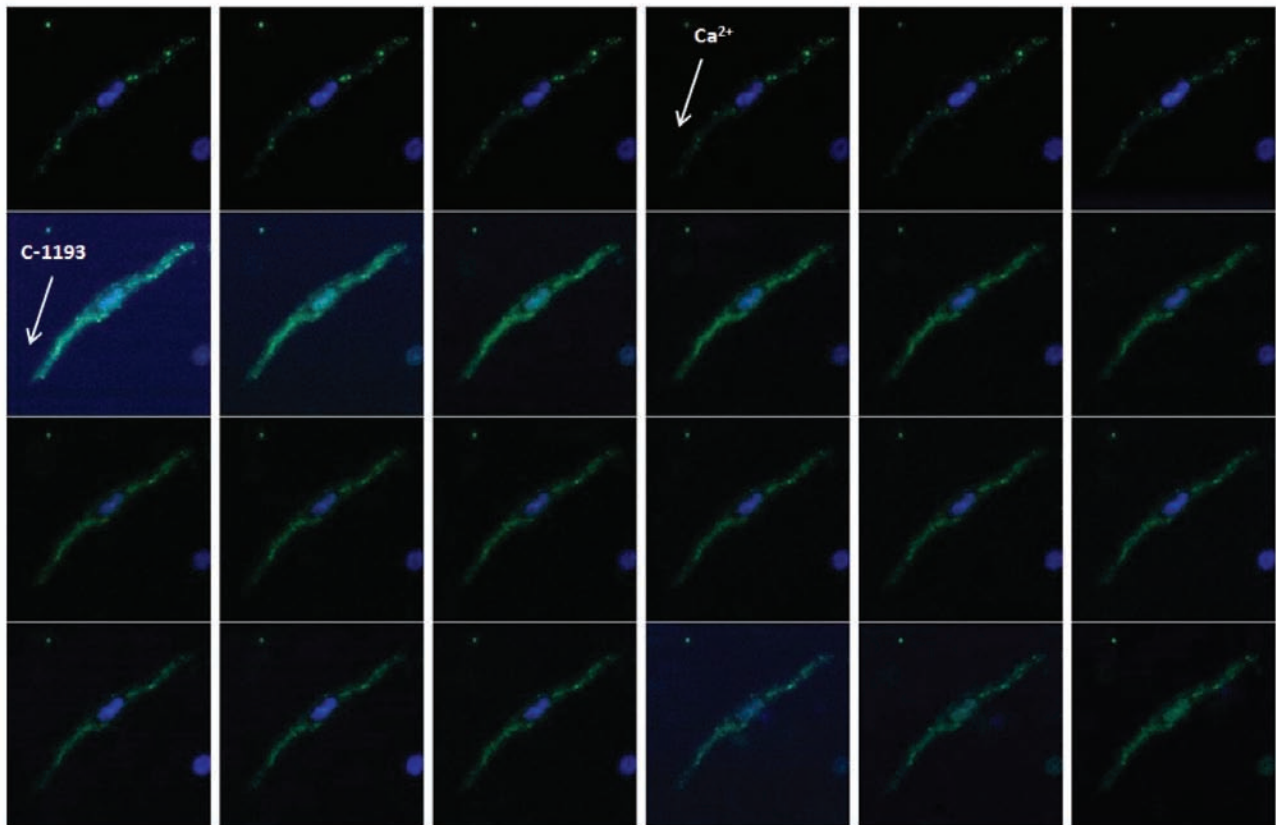


Fig. 5. The series of consecutive photographs of smooth muscle cells using the scanning confocal microscope. The asterisks indicate the moments of introducing Ca^{2+} ($1 \mu\text{M}$) and thiacalix[4]arene C-1193 ($20 \mu\text{M}$). The results of the typical experiment are presented

there was a decrease in the amplitude of contractions on average down to $72.3 \pm 9.1\%$ ($n = 6$, $P < 0.05$); against the background of the increase in the concentration of C-1193 up to $100 \mu\text{M}$, the amplitude was $63.1 \pm 8.4\%$ on average ($n = 6$, $P < 0.05$) (Fig. 6). Also, against the background of C-1193, there were some changes in single spontaneous contractions – an enhanced manifestation of complex contractions with the increase in the concentration of the mentioned compound.

The method of complex mechanokinetic analysis was applied to specific spontaneous contractions (in control and under the effect of C-1193) [30]. It was found that against the background of both investigated concentrations of thiacalix[4]arene C-1193, the indices of temporal parameters of amplitude (τ_{max}) and time of the mechanogram, when the maximal velocity of the relaxation was observed (τ_{R}), remained at the level of the control (Fig. 7, A), whereas the temporal parameter of achieving the maximal velocity of the contraction (τ_{C}) decreased reliably.

It was also found that under the effect of C-1193, the force (F_{max} , F_{C} and F_{R}) parameters decreased considerably and to the same degree (Fig. 7, B); for instance, if C-1193 was present in the washing solution in the concentration of $10 \mu\text{M}$, on average these amounted to 72.3, 68.0 and 69.4% regarding the corresponding control parameters, accepted as 100%. When the concentration of C-1193 increased by one order (up to $100 \mu\text{M}$), these indices tended to decrease further, amounting to 63.1, 49.0, and 60.2%, respectively.

The mechanokinetic analysis demonstrated that under the effect of thiacalix[4]arene C-1193, the impulse parameters decreased considerably, tending towards dose dependence. For instance, against the background of $10 \mu\text{M}$ C-1193, the values of the parameters I_{C} , I_{R} , and I_0 were 54.1, 65.5, and 63.3%, respectively, regarding the control values, whereas at the effect of $100 \mu\text{M}$ of this compound, the average values of these indices were 47.7, 60.4, and 57.1%, respectively.

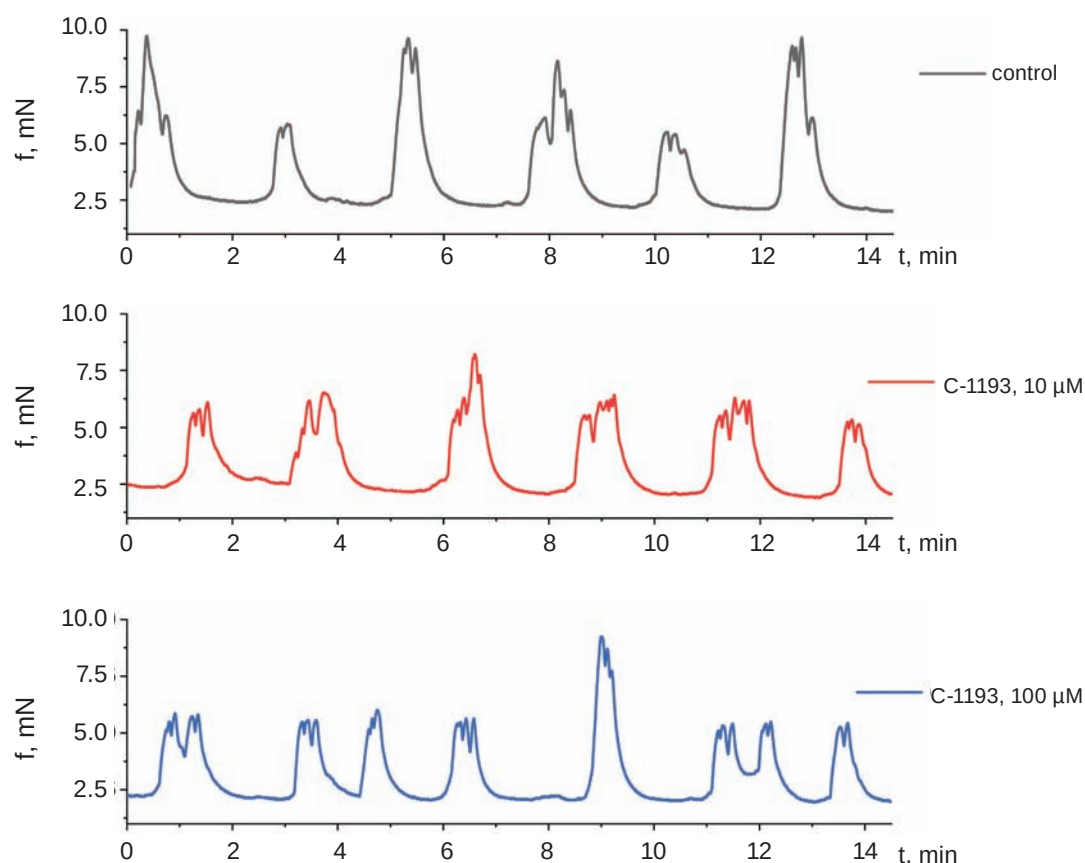


Fig. 6. The spontaneous contractile activity of longitudinal smooth muscles in uterine horns of rats in control and under the effect of thiacalix[4]arene C-1193 (10 and 100 μM). Typical isometric mechanograms are presented

Also, under the effect of C-1193, there was a considerable decrease in the velocities of the phases of contraction (V_C) and relaxation (V_R) with the tendency towards dose-dependence (Fig. 7, D). For instance, against the background of 10 μM of this compound, the values of parameters V_C and V_R on average were 72.1 and 70.0%, respectively, whereas under the effect of 100 μM C-1193 the corresponding indices were on average 69.3 and 55.4% regarding the control. It should be noted that the decrease in the velocity parameters of spontaneous contractions cannot be considered specific in terms of Ca^{2+} -transporting systems of myocytes, since the norm-setting of V_C and V_R regarding the contraction amplitude does not have statistically significant differences from the normalized maximal velocities of the phases of contraction and relaxation in control.

Therefore, in the following series of experiments, we investigated the effect of thiacalix[4]arene C-1193 on the contractile reactions of rat myometrium, induced via the pathways of electro- and phar-

macomechanical coupling of excitation-contraction. The depolarization of PM in smooth muscle cells by high-potassium solution (80 mM) was used as an adequate model of electromechanical coupling; the pharmacomechanical coupling was studied on the example of contractions, induced by the uterotonic hormone oxytocin (0.1 IU), the neurotransmitter of parasympathetic nervous system acetylcholine (10 μM) and the selective agonist of muscarinic acetylcholine receptors of M3-type, cevimeline (100 μM).

Against the background of calix[4]arene C-1193 (10 μM), there was an increase in the amplitude of contractions, activated by hyperkalemic depolarization of PM in smooth muscle cells (Fig. 8). In these conditions, the value of the phasic component increased up to 126.1% on average ($P < 0.05$, $n = 5$), there were also tendencies towards the increase in the tonic component of contractions.

The method of kinetic analysis [31] was used to determine that thiacalix[4]arene C-1193 activated

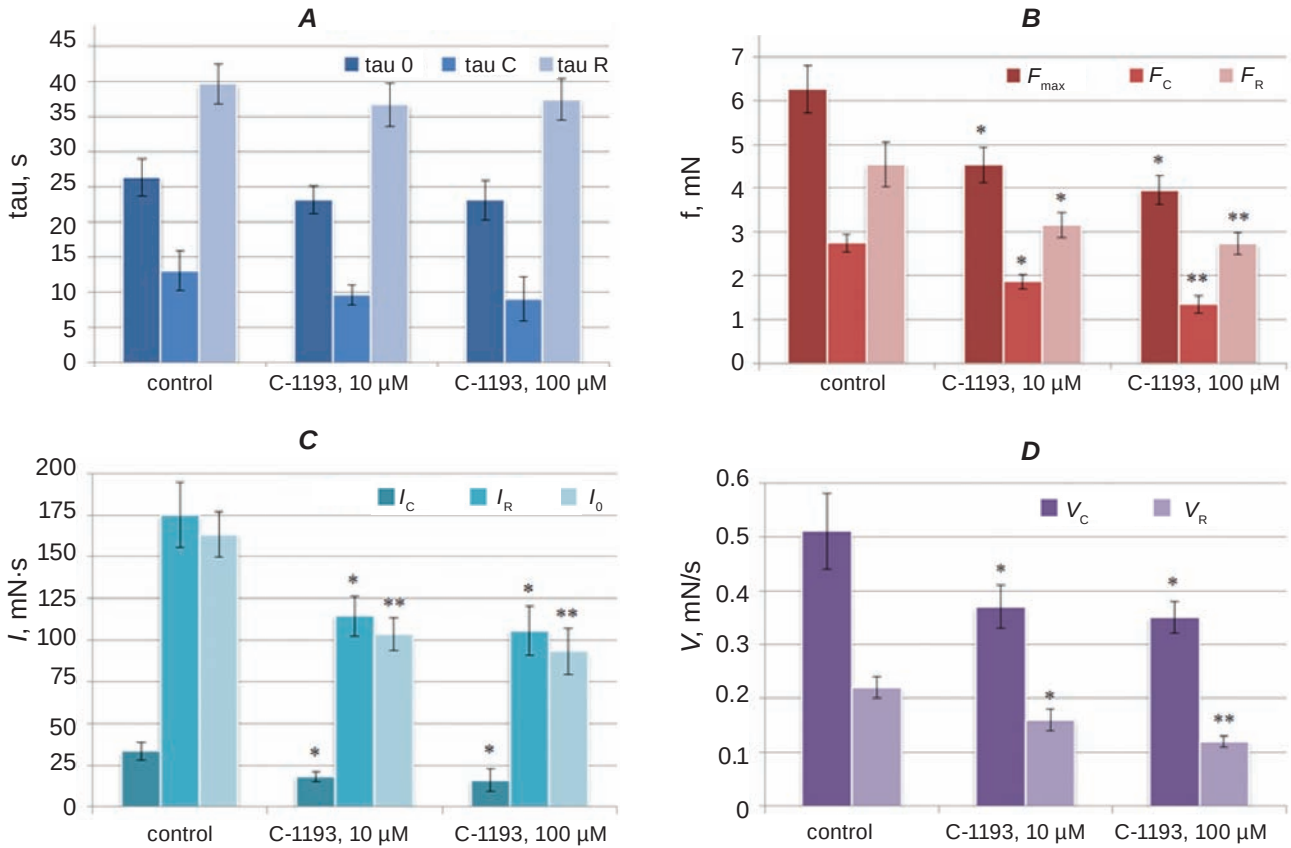


Fig. 7. The parameters of the spontaneous contractile activity of rat myometrium in the control and under the effect of thiocalix[4]arene C-1193 (10 and 100 μM): **A** – time parameters (τ_0 , τ_C and τ_R); **B** – force parameters (F_{max} , F_C and F_R); **C** – impulse parameters (I_0 , I_C and I_R); **D** – velocity parameters (V_C and V_R); $n = 6$; * $P < 0.05$ and ** $P < 0.01$ – the difference is reliable as compared to the control

the process of accelerating the force of contractions, induced by the application of the high-potassium solution, in a considerable way: the parameter V_{nc} was 165.9% on average ($P < 0.01$, $n = 6$). Also, regardless of the increased tonic component, there was a considerable increase in the normalized maximal velocity of the relaxation phase V_{nr} (on average up to 155.9% regarding the control, $P < 0.01$, $n = 6$) (Fig. 8, A and Fig. 9).

Also, under preliminary incubation of smooth muscle preparations of rat uterus with thiocalix[4]arene C-1193, there were changes in the oxytocin-induced contractions: the amplitude of phasic contractions tended to increase, and their tonic components increased considerably on average up to 121.8% ($P < 0.05$, $n = 6$) (Fig. 8, B and 9).

Noteworthy are considerable changes in the kinetics of oxytocin-induced contractions, caused by thiocalix[4]arene C-1193 (10 μM): similar to the HPS-induced contractions, in this case, there was

a considerable increase in the normalized maximal velocity of the contraction phase V_{nc} (on average up to 177.5%, $P < 0.01$, $n = 6$). As for the normalized maximal velocity of the relaxation phase V_{nr} , there was a decrease down to 88.6% on average regarding the control, $P < 0.05$, $n = 6$) (Fig. 8, A and Fig. 9).

Also, against the background of thiocalix[4]arene C-1193 (10 μM), there was an increase in the amplitude of contractions, activated by the exogenous application of the neurotransmitter acetylcholine (Fig. 8, C). Against the background of C-1193, these contractions were characterized by the increase in the phasic component up to 130.2% on average ($P < 0.05$, $n = 6$); there were tendencies towards the increase in the tonic component.

The method of kinetic analysis [31] was used to determine that thiocalix[4]arene C-1193 activated the process of accelerating the force of acetylcholine-induced contractions: the parameter V_{nc} was 119.5% on average ($P < 0.05$, $n = 6$). Also, in these conditions,

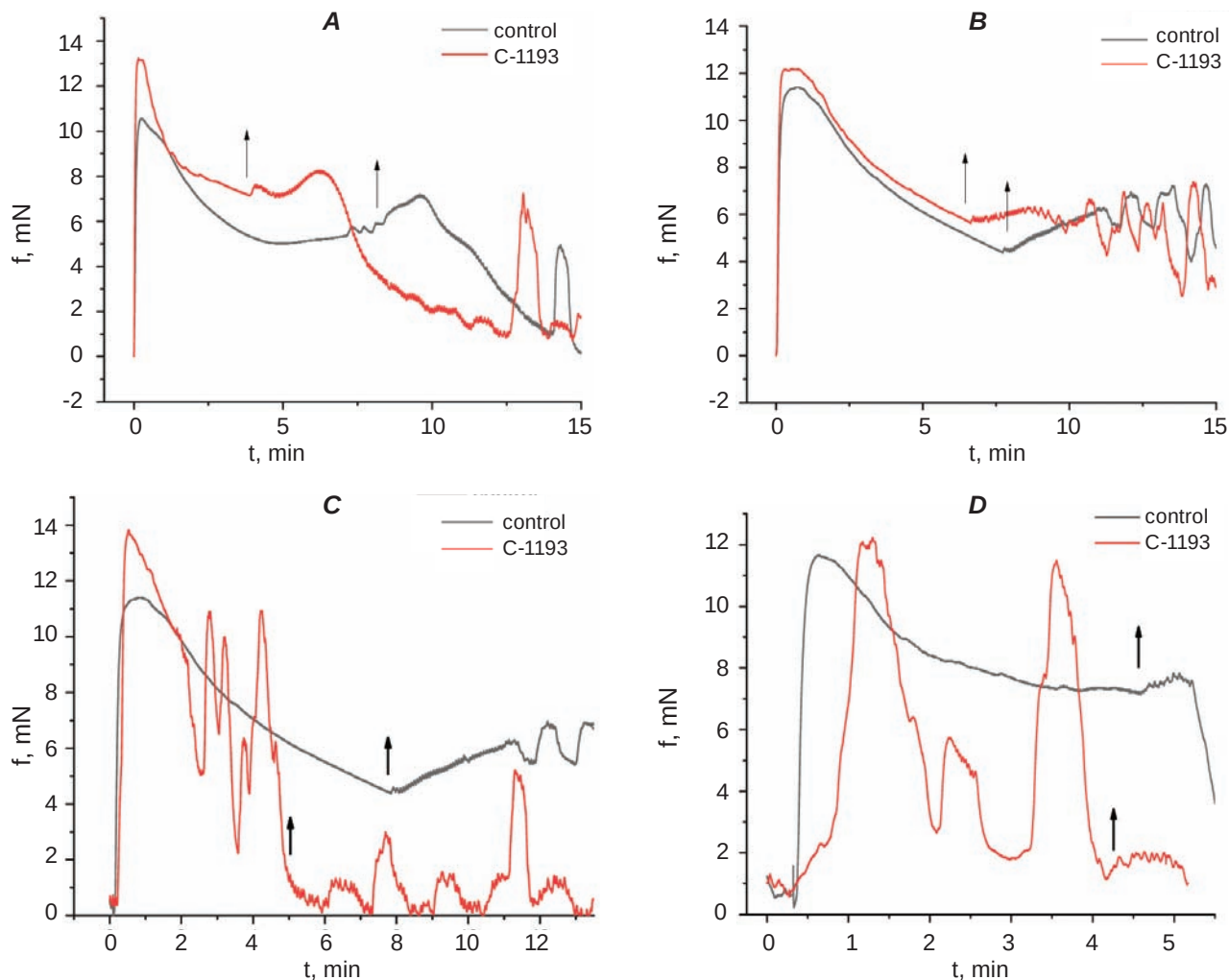


Fig. 8. The induced isometric contractions of longitudinal smooth muscles of rat uterus in the control and under the effect of thiacalix[4]arene C-1193 ($10 \mu\text{M}$, the duration of the previous incubation – 30 min): **A** – contractions, activated with high-potassium solution (80 mM); **B** – contractions, activated with oxytocin (0.1 IU); **C** – contractions, activated with acetylcholine ($10 \mu\text{M}$); **D** – contractions, activated with cevimeline ($100 \mu\text{M}$). The moment of washing muscle preparations with normal Krebs solution is shown with asterisks. Typical mechanograms are presented

there was a considerable decrease in the normalized maximal velocity of the relaxation phase V_{nr} (on average down to 72.4% regarding the control, $P < 0.05$, $n = 6$). So, under the acetylcholine-induced activation of myometrium contractions against the background of C-1193, there generally were the effects, similar to those for oxytocin-activated contractions.

In the following stage, we studied the contractile activity of myometrium, activated by the selective agonist of muscarinic M3-cholinoreceptors, cevimeline ($100 \mu\text{M}$). Under the preliminary incubation of smooth muscle preparations of rat uterus with thiacalix[4]arene C-1193, there was a considerable

increase in the phasic contractions (on average up to 190.2%, $P < 0.01$, $n = 6$) (Fig. 8, D). In these conditions, contrary to acetylcholine-induced contractions, there was a decrease in the normalized maximal velocity of the contraction phase V_{nc} (on average down to 76.3%, $P < 0.05$, $n = 6$), whereas the normalized maximal velocity of the relaxation phase V_{nr} remained on the level of the control (104.2% regarding the control, $P > 0.05$, $n = 6$).

We can also foresee the cellular mechanisms, by which the blocking of Na^+, K^+ -ATPase under the effect of thiacalix[4]arene C-1193 leads to the increase in the intracellular concentration of Ca^{2+}

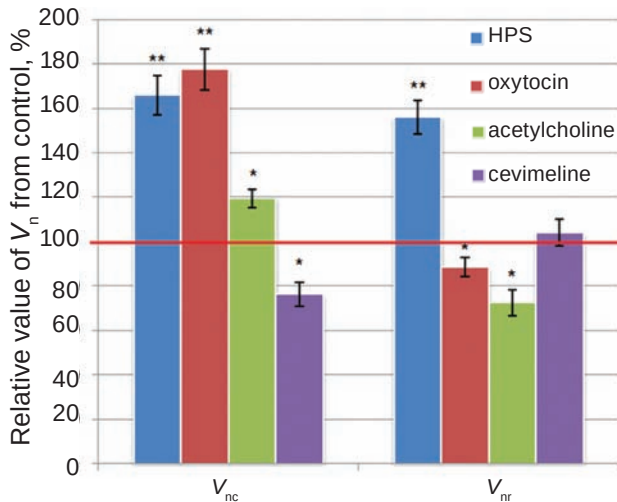


Fig. 9. The modulation of the normalized maximal velocities of the phases of contraction (V_{nc}) and relaxation (V_{nr}) of isometric contractions in myometrium, activated with high-potassium solution (HPS, 80 mM), oxytocin (0.1 IU), acetylcholine (10 μ M) and cevimeline (100 μ M) using thiocalix[4]arene C-1193 (10 μ M). The relevant indices of contractions in the control are accepted as 100%. $n = 6$; * $P < 0.05$ and ** $P < 0.01$ – the difference is reliable as compared to the control

ions in myocytes and the changes in the contractile activity of pluricellular preparations of the longitudinal SM of rat uterus.

There is no doubt that in the excitable tissues, the functioning of Na^+, K^+ -ATPase in PM fulfills the function of maintaining the excitability [37]. In the myocytes of the uterus, this enzyme creates the gradients of Na^+ and K^+ ions and Ca^{2+} ions (due to the formation of gradient Na^+ , which is a driving force for the system of secondary active ion transport – $\text{Na}^+, \text{Ca}^{2+}$ -exchanger) [38-40]. In the non-pregnant myometrium of rats, the isoforms of all three types of subunits are expressed: α_1 (dominating) and α_2 ; β_1 and β_3 ; FXYD1 [38]. It is important that the expression level for some isoforms of α - and β -subunits in the myometrium tissue changes considerably throughout the pregnancy, thus ensuring the optimal level of excitability for fetus bearing and delivery [38, 41].

The known inhibitor of Na^+, K^+ -ATPase of PM, ouabain, which binds to α -subunit of the enzyme, induces the effects at the level of the integral tissue of myometrium, changing the contractile function and causing the increase in the frequency, and in some cases, in the force of spontaneous contractions

[38, 42]. To understand the concentration effects of ouabain (and other blockers of Na^+, K^+ -ATPase in PM), it is relevant that the sensitivity to inhibiting different isoforms of α -subunits of Na^+, K^+ -ATPase differs considerably; in particular, for low-sensitive α_1 -isoform, the effective concentration of ouabain is considered to be 100 μ M, whereas for α_2 -isoform – 10 μ M [43, 44]. It is also relevant that α_2 -isoform performs the signalling function in an ouabain-sensitive way, mediated by Src-kinase, modulating the reaction of SMC on the application of agonists of metabotropic receptors [43-46].

In our previous studies, we showed that, similarly to ouabain, calix[4]arene C-99 (both in the concentration of 10 μ M) induced the activation of spontaneous contractions in the rat myometrium and modulated the mechanokinetics of contractile reactions on HKS-induced depolarization of PM and application of the agonists of metabotropic receptors (oxytocin and acetylcholine) [42]. In this study, we revealed that thiocalix[4]arene C-1193 (10 μ M) caused the increase in the frequency against the background of the decrease in the amplitude of spontaneous contractions in the myometrium and modulation of induced contractile reactions. It should be noted that in many cases (in terms of the increase in the frequency of spontaneous contractions, the direction of the change in parameters of normalized maximal velocities of the phases of contraction and relaxation, induced by acetylcholine and oxytocin), the effects, previously registered by us against the background of C-99 and C-1193, are in agreement [42].

At the same time, noteworthy is the fact that when thiocalix[4]arene C-1193 (10 and 100 μ M) was present in the washing solution, it induced a considerable decrease in the amplitude of spontaneous contractions (Fig. 6). This difference may be explained with the consideration of the data of sustained increase in the level of cytosolic Ca^{2+} in myocytes under the application of C-1193 (Fig. 4 and 5). It is known that under the increase in the concentration of Ca^{2+} in the near-membrane space of smooth muscle cells, there is the activation of Ca^{2+} -sensitive ion channels, in particular, Ca^{2+} -sensitive K^+ -channels of high permeability ($\text{K}_{\text{Ca}1.1}$), which are expressed in the myocytes of the uterus of rats [47-49]. This probable increase in the initial K^+ -current may condition the decrease in the amplitude of spontaneous contractions of the myometrium under the effect of C-1193.

Conclusions. All obtained results are important for understanding and subsequent investigation of mechanisms of Na^+, K^+ -ATPase inhibition by calixarene C-1193 and can be a foundation for the creation of new more effective inhibitors of mentioned enzyme and uterotonics for medicine, based on the calixarene core.

In general, we expect that the experimental data of using thiacalix[4]arene C-1193 – a selective and effective inhibitor of Na^+, K^+ -ATPase may be relevant for the research on determining the membrane mechanisms of cation exchange in smooth muscles, in particular, for the investigation of the role of the plasma membrane in ensuring the electro- and pharmacomechanical coupling in them, and in the regulation of ion homeostasis in smooth muscle cells.

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

Funding. This work was financially supported by the grants, the state registration numbers: 0119U002508, 0123U100894.

ТІАКАЛІКС[4]АРЕН C-1193 – АФІННИЙ ІНГІБІТОР НАТРІЄВОЇ ПОМПИ, ЗБІЛЬШУЄ ВНУТРІШНЬОКЛІТИННУ КОНЦЕНТРАЦІЮ ІОНІВ Ca^{2+} ТА МОДИФІКУЄ СКОРОТЛИВУ АКТИВНІСТЬ МІОМЕТРІЯ

Т. О. Веклич¹✉, С. О. Черенок²,
О. В. Цимбалюк³, О. А. Шкрабак¹,
С. О. Карахім¹, А. І. Селіхова²,
В. І. Кальченко², С. О. Костерін¹

¹Інститут біохімії ім. О. В. Палладіна
НАН України, Київ;

✉e-mail: veklich@biochem.kiev.ua;

²Інститут органічної хімії НАН України, Київ;

³Навчально-науковий інститут високих
технологій, Київський національний
університет імені Тараса Шевченка, Україна

З використанням методів ензиматичного та кінетичного аналізу було продемонстровано, що тіакалікс[4]арен-біс-гідроксиметилфосфонова кислота C-1193 має більш ефективну інгібіторну дію на Na^+, K^+ -АТРазну активність у плазматичній мембрані клітин міометрія ($I_{0,5} = 42,1 \pm 0,6$ нМ) порівняно з калікс[4]арен-

біс-гідроксиметилфосфоною кислотою C-99, і практично не впливає на питомі активності інших АТРаз, що локалізовані у цій субклітинній структурі. Методом конфокальної мікроскопії з використанням Ca^{2+} -чутливого флуоресцентного зонду fluo-4 показано, що тіакалікс[4]арен C-1193 зумовлює зростання внутрішньоклітинної концентрації Ca^{2+} в іммобілізованих міоцитах матки. Тензометричними дослідженнями продемонстровано, що C-1193 (10 та 100 мкМ) модифікує механокінетику спонтанних ізометричних скорочень міометрія. На тлі використання C-1193 (10 мкМ) спостерігали збільшення ізометричних фазних скорочень, спричинених електромеханічним (деполяризація гіперкалієвим розчином) та фармакомеханічним спряженням (за умов дії утеротонічного гормону окситоцину, нейромедіатора ацетилхоліну та селективного агоніста мускаринових ацетилхолінових рецепторів М3-типу цевімеліну). Передбачається, що отримані експериментальні дані, які були одержані з використанням тіакалікс[4]арену C-1193 – селективного та ефективного інгібітора Na^+, K^+ -АТрази, можуть мати значення для з'ясування мембранних механізмів катіонного обміну у гладеньких м'язах, зокрема, під час вивчення ролі плазматичної мембрани в забезпеченні електро- та фармакомеханічного спряження в них, а також у регуляції іонного гомеостазу в міоцитах.

Ключові слова: $\text{Ca}^{2+}, \text{Mg}^{2+}$ -АТРаза, плазматична мембрана, гладеньком'язові клітини, міометрій, ензиматичний гідроліз АТР, кінетичні параметри, мультиклітинні препарати гладеньких м'язів, тіакалікс[4]арени.

References

1. Buxton IL. Regulation of uterine function: a biochemical conundrum in the regulation of smooth muscle relaxation. *Mol Pharmacol.* 2004; 65(5): 1051-1059.
2. Woodcock NA, Taylor CW, Thornton S. Effect of an oxytocin receptor antagonist and rho kinase inhibitor on the $[\text{Ca}^{2+}]_i$ sensitivity of human myometrium. *Am J Obstet Gynecol.* 2004; 190(1): 222-228.
3. Arakawa TK, Mlynarczyk M, Kaushal KM, Zhang L, Ducsay CA. Long-term hypoxia alters calcium regulation in near-term ovine myometrium. *Biol Reprod.* 2004; 71(1): 156-162.

4. Biser PS, Thayne KA, Fleming WW, Taylor DA. Na⁺, K⁺ ATPase α -subunit isoform distribution and abundance in guinea-pig longitudinal muscle/myenteric plexus after exposure to morphine. *Brain Res.* 2002; 931(2): 186-193.
5. Geering K. FXYD proteins: new regulators of Na-K-ATPase. *Am J Physiol Renal Physiol.* 2006; 290(2): F241-F250.
6. Dos Santos L, Xavier FE, Vassallo DV, Rossoni LV. Cyclooxygenase pathway is involved in the vascular reactivity and inhibition of the Na⁺, K⁺-ATPase activity in the tail artery from L-NAME-treated rats. *Life Sci.* 2003; 74(5): 613-627.
7. Jain D, Chhabra SK, Paj HG. Effect of sensitization on membrane ion fluxes & intracellular calcium in guineapigs. *Indian J Med Res.* 2004; 120(6): 534-541.
8. Isenovic ER, Jacobs DB, Kedeas MH, Sha Q, Milivojevic N, Kawakami K, Gick G, Sowers JR. Angiotensin II regulation of the Na⁺ pump involves the phosphatidylinositol-3 kinase and p42/44 mitogen-activated protein kinase signaling pathways in vascular smooth muscle cells. *Endocrinology.* 2004; 145(3): 1151-1160.
9. Kalchenko VI, Rodik RV. Supramolecular nanomachines and smart materials. *Visn Nac Acad Nauk Ukr.* 2017; (1): 82-88.
10. Kalchenko VI. Functional calixarene nanostructures. *Theor Exp Chem.* 2018; 54(2): 74-84.
11. Kosterin SO, Kalchenko VI, Veklich TO, Babich LG, Shlykov SG. Calixarenes as modulators of ATP-hydrolyzing systems of smooth muscles. K.: Science opinion, 2019. 256 p.
12. Rodik RV, Boyko VI, Kalchenko VI. Calixarenes in Biotechnology and Bio-Medical Researches. *Front Med Chem.* 2016; 8: 206-301.
13. Shkrabak OA, Veklich TO, Rodik RV, Kalchenko VI, Kosterin SO. Inhibition of plasma membrane Ca²⁺,Mg²⁺-ATPase by calixarene sulfonylamidines. Structure-activity relationship. *Ukr Biochem J.* 2022; 94(4): 18-35.
14. Veklich TO, Labyntseva RD, Shkrabak OA, Tsymbaluk OV, Rodik RV, Kalchenko VI, Kosterin SO. Inhibition of Na⁺,K⁺-ATPase and activation of myosin ATPase by calix[4]arene C-107 cause stimulation of isolated smooth muscle contractile activity. *Ukr Biochem J.* 2020; 92(1): 21-30.
15. Veklich TO, Shkrabak OA, Rodik RV, Boyko VI, Kalchenko VI, Kosterin SO. Spatial structure of the calixarene-aminophosphonic acids is important for their inhibition of the Na⁺,K⁺-ATPase activity in plasma membrane of smooth muscle cells. *Ukr Biokhim Zhurn.* 2010; 82(1): 21-33. (In Ukrainian).
16. Veklich TO, Kosterin SO, Rodik RV, Cherenok SO, Boyko VI, Kalchenko VI. Effect of calixarene-phosphonic acid on Na⁺,K⁺-ATPase activity in plasma membranes of the smooth-muscle cells. *Ukr Biokhim Zhurn.* 2006; 78(1): 70-86. (In Ukrainian).
17. Veklich TO, Shkrabak OA, Cherenok SO, Kalchenko VI, Kosterin SO. Comparative investigation of the effect of calix[4]arene C-99 and its analogs on Na⁺,K⁺-ATPase activity of uterus myocyte plasma membrane. *Ukr Biokhim Zhurn.* 2012; 84(6): 49-57. (In Ukrainian).
18. Arduini A, Fanni S, Manfredi G, Pochini A, Ungaro R, Sicuri A, Ugozzoli FJ. Direct Regioselective Formylation of Tetraalkoxycalix[4]arenes Fixed in the Cone Conformation and Synthesis of New Cavitands. *J Org Chem.* 1995; 60(5): 1448-1453.
19. Yang W, Wang W, Guo R, Gong L, Gong S. Convenient Direct Syntheses of Selectively para-Substituted Di-, Tri- and Tetra-Formylated Thiocalix[4]arenes. *Eur J Org Chem.* 2012; 3326-3330.
20. Sekine M, Okimoto K, Yamada K, Hata T. Silyl phosphites. 15. Reactions of silyl phosphites with α -halo carbonyl compounds. Elucidation of the mechanism of the Perkow reaction and related reactions with confirmed experiments. *J Org Chem.* 1981; 46(10): 2097-2107.
21. Cherenok SO, Yushchenko OA, Tanchuk VYu, Mischenko IM, Samus NV, Ruban OV, Matvieiev Y, Karpenko JA, Kukhar VP, Vovk AI, Kalchenko VI. Calix[4]arene- α -hydroxyphosphonic acids. Synthesis, stereochemistry, and inhibition of glutathione S-transferase. *Arkivoc.* 2012; (IV): 278-298.
22. Veklich TO, Kosterin SO. Comparative study of properties of Na⁺,K⁺-ATPase and Mg²⁺-ATPase of the myometrium plasma membrane. *Ukr Biokhim Zhurn.* 2005; 77(2): 66-75. (In Ukrainian).
23. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of

- protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72(1-2): 248-254.
24. Mollard P, Mironneau J, Amedee T, Mironneau C. Electrophysiological characterization of single pregnant rat myometrial cells in short-term primary culture. *Am J Physiol.* 1986; 250(1 Pt 1): C47-C54.
 25. Valente RC, Capella LS, Monteiro RQ, Rumjanek VM, Lopes AG, Capella MAM. Mechanisms of ouabain toxicity. *FASEB J.* 2003; 17(12): 1700-1702.
 26. Wang H, Haas M, Liang M, Cai T, Tian J, Li S, Xie Z. Ouabain assembles signaling cascades through the caveolar Na⁺/K⁺-ATPase. *J Biol Chem.* 2004; 279(17): 17250-17259.
 27. Rathbun WB, Betlach MV. Estimation of enzymically produced orthophosphate in the presence of cysteine and adenosine triphosphate. *Anal Biochem.* 1969; 28(1): 436-445.
 28. Mazur IuI, Veklich TO, Shkrabak OA, Mohart MA, Demchenko AM, Gerashchenko IV, Rodik RV, Kalchenko VI, Kosterin SO. Selective inhibition of smooth muscle plasma membrane transport Ca²⁺, Mg²⁺-ATPase by calixarene C-90 and its activation by IPT-35 compound. *Gen Physiol Biophys.* 2018; 37(2): 223-231.
 29. Magocsi M, Penniston JT. Ca²⁺ or Mg²⁺ nucleotide phosphohydrolases in myometrium: two ectoenzymes. *Biochim Biophys Acta.* 1991; 1070(1): 163-172.
 30. Kosterin S, Tsymbalyuk O, Holden O. Multiparameter analysis of mechanokinetics of the contractile response of smooth muscles. *Series Biomechanics.* 2021; 35(1): 14-30.
 31. Burdyga TV, Kosterin SA. Kinetic analysis of smooth muscle relaxation. *Gen Physiol Biophys.* 1991; 10(6): 589-598.
 32. Lang J, Dvořáková H, Bartošová I, Lhotak P, Stibor I, Hrabal R. Conformational flexibility of a novel tetraethylether of thiocalix[4]arene. A comparison with the "classical" methylene-bridged compounds. *Tetrahedron Lett.* 1999; 40(2): 373-376.
 33. Kasyan O, Healey ER, Drapailo A, Zaworotko M, Cecillon S, Coleman AW, Kalchenko V. Synthesis, Structure and Selective Upper Rim Functionalization of Long Chained Alkoxythiocalix[4]arenes. *J Incl Phenom Macrocycl Chem.* 2007; 58: 127-132.
 34. Kosterin SO, Babich LG, Shlykov SG, Danylovyh IuV, Veklich TO, Mazur YuYu. Biochemical properties and regulation of smooth muscle cell Ca²⁺-transporting systems. K.: Science opinion, 2016. 210 p.
 35. Noblel D, Herchuelz A. Role of Na/Ca exchange and the plasma membrane Ca²⁺-ATPase in cell function. Conference on Na/Ca exchange. *EMBO Rep.* 2007; 8(3): 228-232.
 36. Man'ko VV. Methodological approaches to investigation of Na⁺-Ca²⁺ exchange in exocrine secretory cells. *Ukr Biokhim Zhurn.* 2006; 78(1): 43-62. (In Ukrainian).
 37. Clausen T. Na⁺-K⁺ pump regulation and skeletal muscle contractility. *Physiol Rev.* 2003; 83(4): 1269-1324.
 38. Floyd RV, Mobasher A, Wray S. Gestation changes sodium pump isoform expression, leading to changes in ouabain sensitivity, contractility, and intracellular calcium in rat uterus. *Physiol Rep.* 2017; 5(23): e13527.
 39. Blaustein MP, Juhaszova M, Golovina VA, Church PJ, Stanley EF. Na/Ca exchanger and PMCA localization in neurons and astrocytes: functional implications. *Ann N Y Acad Sci.* 2002; 976: 356-366.
 40. Golovina VA, Song H, James PF, Lingrel JB, Blaustein MP. Na⁺ pump alpha 2-subunit expression modulates Ca²⁺ signaling. *Am J Physiol Cell Physiol.* 2003; 284(2): C475-C486.
 41. Floyd R, Mobasher A, Martin-Vasallo P, Wray S. Na,K-ATPase isoforms in pregnant and nonpregnant rat uterus. *Ann N Y Acad Sci.* 2003; 986: 614-616.
 42. Tsymbalyuk OV. Comparative analysis of the mechanokinetics of contractile activity of myometrium smooth muscles under calixarene C-99 and ouabain action. *Studia Biologica.* 2014; 8(1): 63-72.
 43. Matchkov VV, Moeller-Nielsen N, Dam VS, Nourian Z, Briggs Boedtkjer DM, Aalkjaer C. The α2 isoform of the Na,K-pump is important for intercellular communication, agonist-induced contraction, and EDHF-like response in rat mesenteric arteries. *Am J Physiol Heart Circ Physiol.* 2012; 303(1): H36-H46.
 44. Hangaard L, Bouzinova EV, Staehr C, Dam VS, Kim S, Xie Z, Aalkjaer C, Matchkov VV. Na-K-ATPase regulates intercellular communication in the vascular wall via cSrc kinase-dependent connexin43 phosphorylation. *Am J Physiol Cell Physiol.* 2017; 312(4): C385-C397.

45. Zhang L, Aalkjaer C, Matchkov VV. The Na,K-ATPase-Dependent Src Kinase Signaling Changes with Mesenteric Artery Diameter. *Int J Mol Sci*. 2018; 19(9): 2489.
46. Tsymbalyuk OV, Kosterin SO. Na⁺,K⁺-ATPase, endogenous cardiotonic steroids and their transducing role. *Ukr Biokhim Zhurn*. 2012; 84(1): 5-17. (In Ukrainian).
47. Novakovic R, Ilic B, Beleslin-Cokic B, Radunovic N, Heinle H, Scepanovic R, Gojkovic-Bukarica L. The effect of resveratrol on contractility of non-pregnant rat uterus: the contribution of K⁺ channels. *J Physiol Pharmacol*. 2013; 64(6): 795-805.
48. Piedras-Rentería E, Stefani E, Toro L. Potassium currents in freshly dispersed myometrial cells. *Am J Physiol*. 1991; 261(2 Pt 1): C278-C284.
49. Shmygol A, Noble K, Wray S. Depletion of membrane cholesterol eliminates the Ca²⁺-activated component of outward potassium current and decreases membrane capacitance in rat uterine myocytes. *J Physiol*. 2007; 581(Pt 2): 445-456.