Biol. Stud. 2025, 19(1), 15–34 • doi: https://doi.org/10.30970/sbi.1901.816
www.http://publications.lnu.edu.ua/journals/index.php/biology

UDC: 577.152.3+544.147+544.176+544.168

KINETIC REGULARITIES OF THIACALIX[4]ARENE C-1193 ACTION ON Na^+, K^+-ATPase ACTIVITY OF THE PLASMA MEMBRANE AND CONTRACTILE ACTIVITY OF THE MYOMETRIUM

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Tsymbalyuk, O., Veklich, T., Maliuk, O., Cherenok, S., Kalchenko, V., & Kosterin, S. (2025). Kinetic regularities of thiacalix[4]arene C-1193 action on Na^+, K^+-ATPase activity of the plasma membrane and contractile activity of the myometrium. *Studia Biologica*, 19(1), 15–34. doi:10.30970/sbi.1901.816

Background. Na^+, K^+-ATPase (sodium pump) is an electrogenic Ca^{2+}-independent Mg^{2+}, Na^+, K^+-ATP-dependent transporting system of plasma membrane (PM), which conducts active transfer of univalent ions of Na and K and thus maintains their electrochemical gradients, required for normal functioning of the cell. It was proven that in some widesperad pathologies the activity of the sodium pump is disrupted. Therefore, the search for effectors – selective inhibitors and activators that would be able to specifically affect Na^+, K^+-ATPase, restoring its activity in pathological conditions, is very promising. The aim of this study was to investigate the action of thiacalix[4]arene C-1193 on the dependence of Na^+, K^+-ATPase activity of PM of myometrium cells on the concentration of ATP and Mg ions, as well as on the isotonic contractile activity of the myometrium.

Materials and Methods. The experiments were conducted using outbred white non-pregnant rats. The inhibitory action of thiacalix[4]arene C-1193 (25,27-dibutoxythiacalix[4]arene-bis-hydroxymethylphosphonic acid) on the kinetic traits of Na^+, K^+-ATPase activity was studied in the experiments, conducted using the suspension of perforated plasma membranes of the myometrium cells. The registration of the contractile activity in the preparations of longitudinal smooth muscles of uterine horns with preserved endothelium was done in the isotonic mode. The study of the contractile activity of muscle preparations was carried out using mechanokinetic analysis methods.

Results. The study demonstrated that while inhibiting Na^+, K^+-ATPase, thiacalix[4]arene C-1193 did not change the kinetic parameters (K_m, n_H) of the dependence of the reaction velocity on the substrate concentration. Calix[4]arene C-1193 had practically no action on the affinity between Na^+, K^+-ATPase and ATP, which demonstrated the absence of competition between the binding centers for ATP and C-1193. There was no effect on the affinity and cooperative action of Mg ions either. Both cases demonstrated a considerable reduction in the maximal velocity of ATP hydrolysis.

It was found that thiacalix[4]arene C-1193 (in the concentration of 10 μM) modulated the isotonic reactions of pluricellular preparations of myometrium, induced via the pathways of electro- and pharmacomechanic coupling. It was also determined that under the effect of C-1193, there was an increase both in the amplitude of contractions and in the mechanokinetic parameters: contractions (ΔL_{max}, ΔL_C and ΔL_R) and velocities (V_C and V_R). The norm-setting for V_C and V_R regarding the amplitude of contractions at the action of C-1193 resulted in the loss of statistically significant differences between the maximal velocities of the contraction and relaxation phases.

Conclusions. The results of our research indicate that thiacalix[4]arene C-1193, has a non-competitive mechanism of inhibiting Na^+, K^+-ATPase activity and has no specific action on Ca^{2+}-transporting systems of uterine myocytes.

Keywords: myometrium, Na^+,K^+-ATPase, plasma membrane, thiacalix[4]arene C-1193, smooth muscle cells, kinetic traits of ATPase, contractions, mechanokinetic parameters

INTRODUCTION

Na^+, K^+-ATPase (sodium pump) is an electrogenic Ca^{2+}-independent Mg^{2+}, Na^+, K^+-ATP-dependent transporting system of plasma membrane (PM), which conducts active transfer of univalent ions Na and K and thus maintains their electrochemical gradients, required for normal functioning of the cell (ensuring the electric excitability of the nervous and muscle tissues, energy provision for Na^+-dependent secondary active transportation of Ca ions and protons – Na^+-Ca^{2+} and Na^+-H^+ exchange, regulating the cellular volume, etc.) (Amazu *et al*., 2020; Kumari & Rathore, 2020; Gao *et al*., 2021; Contreras *et al*., 2024).

On the one hand, the study of biochemical aspects of regulating the activity of Na^+, K^+-ATPase is of considerable interest for the understanding of ionic, molecular, and membrane mechanisms of control over the functions of different tissues, including muscle tissue. On the other hand, it was proven that in such pathologies as diabetes and ischemia, there is a decrease in the sodium pump activity (Valadares *et al*., 2021). Therefore, the search for reverse effectors – selective inhibitors and activators, capable of targeted action on Na^+, K^+-ATPase, restoring its activity under pathologies, is quite a promising trend from both fundamental and practical standpoints.

In the context of the abovementioned, cyclic oligomers of phenols – calixarenes – can be referred to as modulators. They are of low toxicity (Coleman *et al*., 2008; Al-Ahmary *et al*., 2024) and immunogenicity (Paclet *et al*., 2006; Wojaczyńska *et al*., 2024), and some of them have bactericide, antiviral, antithrombotic, and antitumour properties. The mentioned compounds (at least some of them) demonstrate a membranotrophic effect, penetrate the cell through the plasma membrane easily, and are rather promising in terms of creating novel and highly efficient selective inhibitors and activators

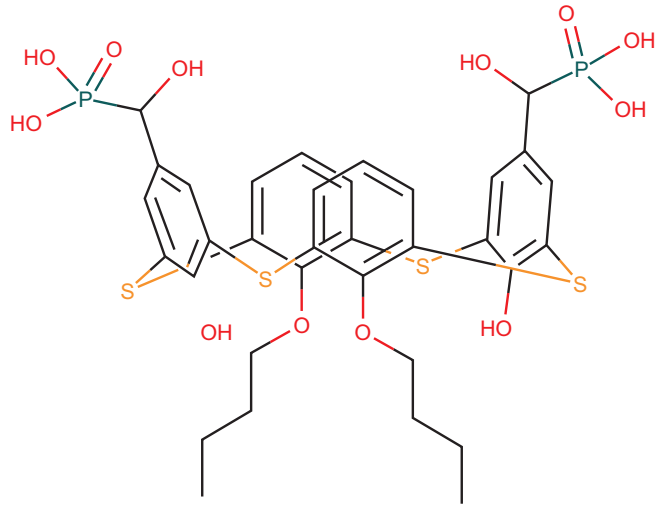
of intracellular biochemical processes because they are capable of reverse modification of the functional activity of some proteins (Pan *et al*., 2021; Mourer *et al*., 2023).

Our previous studies, conducted using the PM preparations of uterine myocytes, demonstrated that thiacalix[4]arene C-1193 efficiently inhibited the enzymatic activity of ouabain-sensitive Na^+, K^+-ATPase, practically without affecting the activity of Mg^{2+}-ATPase, Ca^{2+}-ATPase and Ca^{2+}, Mg^{2+}-ATPase of PM (Veklich *et al*., 2023).

In this study, we aimed to investigate the action of thiacalix[4]arene C-1193 on the dependence of Na^+, K^+-ATPase activity of PM of myometrium cells on the concentration of ATP and Mg ions, as well as on the isotonic contractile activity of the myometrium.

MATERIALS AND METHODS

Thiacalix[4]arene C-1193 (25,27-dibutoxythiacalix[4]arene-bis-hydroxymethylphosphonic acid) (the structure see below) was synthesized and characterized using NMR and infrared spectroscopy methods at the Phosphoranes Chemistry Department of the Institute of Organic Chemistry, the NAS of Ukraine. The method of synthesizing the mentioned thiacalix[4]arene was described previously (Veklich *et al*., 2023).



Thiacalix[4]arene C-1193

The enzymatic studies were conducted at the Muscle Biochemistry Department of the O.V. Palladin Institute of Biochemistry, the NAS of Ukraine.

The PM fraction of smooth muscle cells was isolated from the porcine myometrium as described before (Kondratiuk *et al*., 1986; Veklich & Kosterin, 2005).

The protein content in the membrane fraction was determined by the method of M. Bredford using the reaction with Coomassie reagent – G250 (Bradford, 1976).

The "total" Mg^{2+}, Na^+, K^+-ATPase activity was determined in the PM fraction of myometrium cells as described before (Veklich & Kosterin, 2005), 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 was 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 ethylenediaminetetraacetic acid (EDTA) in the incubation medium ensured the binding of endogenous ions of Ca therein.

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 (Valente *et al.*, 2003; Blaustein & Hamlyn, 2024)).

The amount of P_i reaction product was determined by the method of W. Rathbun and V. Betlach (Rathbun & Betlach, 1969).

The experiments on the investigation of the action of different ATP concentrations (0.01–1 mM) and thiacalix[4]arene C-1193 (10–100 nM) on Na^+, K^+-ATPase activity involved the abovedescribed standard incubation medium with the addition of the aliquote of the ATP solution in the relevant concentration. The concentration of $MgCl_2$ in the standard incubation medium was constant, 3 mM.

The experiments on the action of different concentrations of Mg^{2+} ions (0.01–3 mM) and thiacalix[4]arene C-1193 (10–100 nM) on Na^+, K^+-ATPase activity involved the abovedescribed standard incubation medium with the addition of the aliquote of $MgCl_2$ solution in the relevant concentration. The concentration of ATP in the standard incubation medium was constant, 1 mM. All the experiments involved the use of a concentrated (1 mM) solution of thiacalix[4]arene C-1193 in DMSO, which was further diluted with water to reach the final required concentration.

The kinetic parameters of the action of ATP and Mg^{2+} ions on the enzymatic activity of Na^+, K^+-ATPase were estimated using the concentration dependencies, built in double logarithmic coordinates regarding the linearized equation of Hill $\lg[(V_{max}–V)/V] = n_H \cdot \lg K – n_H \cdot \lg S$, where V is the relative enzymatic activity, V_{max} is the maximal relative enzymatic activity, K is the apparent Michaelis constant or apparent constant of the activation with Mg ions, and S is the concentration of substrate or ion-activator in the incubation medium. The values of the apparent constant of the inhibition K_i with thiacalix[4]arene C-1193 and Hill’s coefficient were estimated using the linearized charts of Hill according to the equation: $\lg[(V_0–V)/V] = n_H \cdot \lg I – n_H \cdot \lg K_i$, where V is the relative enzymatic activity in the absence of an inhibitor and I is the inhibitor concentration 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.

Functional tenzometric experiments. The contractile activity of longitudinal smooth muscle stripes of uterine horns was registered by the tenzometric method in the isotonic mode. Muscle stripes (the average size – 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 preparation was tensed at the rate of 10 mN and left for at least 1 h (until achieving spontaneous contractions of stable amplitude and frequency). The signals were registered with an analogue-to-digital transformer.

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^+ ions in the concentration of 80 mM, was prepared by isotonic replacement of the required amount of Na^+ ions in the initial Krebs solution with the equimolar amount of K^+ ions.

Calix[4]arene C-1193 was preliminarily dissolved in DMSO and added to Krebs solution or HPS in the concentration of 10 μM (the final aliquot of the organic solvent solution was 0.1 % from the total volume of this solution). Oxytocin was added to Krebs solution in the amount, corresponding to the activity of 0.1 IU.

The study of the spontaneous contractile activity in muscle preparations was conducted according to the empirical multiparameter method of the complex mechanokinetic analysis (Kosterin & Tsymbalyuk, 2023). The mechanokinetic curves for the cycles of isotonic contraction-relaxation of myometrium preparations according to the method (Kosterin & Tsymbalyuk, 2023) were linearized (R^2 = 0.96–0.99) in double logarithmic coordinates $\left[\ln\left(\frac{\Delta l_R}{\Delta l_C}\right);\left(1+\frac{\Delta t}{t}\right)\right]$, where Δl_C is a change in the muscle preparation length at the level of contraction phase proper at any time moment $t < \tau_0$, and Δl_R is a change in the muscle preparation length at the level of relaxation phase proper at any time moment $t + \Delta t > \tau_0$ (Δt = const). The linearization charts were used to determine the constants k and n, which were further used to calculate the mechanokinetic parameters: the value of time of the contraction amplitude τ_0; the values of the characteristic times of the contraction (τ_C) and relaxation (τ_R) phases; the legnth of contraction phase at the inflexion point ($t = \tau_C$) ΔL_C and the contraction velocity at this point V_C; the length of contraction phase at the inflexion point ($t = \tau_R$) ΔL_R and the relaxation velocity at this point V_R. We also calculated the energy parameters of spontaneous contractions: the maximum work ΔA_{max} performed by the smooth muscle preparation at the level of the purely isotonic (f = 10 mN) contraction phase for time τ_0; the work performed by the muscle for the characteristic time τ_C; the maximum contraction power N_{max} (at time τ_C) and the average power N_m at the level of the isotonic contraction phase.

In case of contractions induced by HPS and oxytocin, the normalized maximal velocities of the contraction and relaxation phases were estimated by the method of Kosterin and Burdyga (Burdyga & Kosterin, 1991; Tsymbalyuk & Vadzyuk, 2020). According to the method, the phases of contraction and relaxation were separately linearized within the coordinates $\left[\ln\left(\frac{\Delta L_{max}-\Delta L}{L}\right);\ln t\right]$ and the linearized charts were used to determine the parameters τ and n. The values for n and τ were used to estimate the normalized maximal velocities (V_n: V_{nC} – contraction phase and V_{nR} – relaxation phase).

Reagents. The following reagents were used in the experiments: ATP, Hepes, ouabain, acetylcholine, 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.

Statistical analysis. The experimental data were processed by variation statistics methods using Origin 2018 and Excel software packages. The samples were checked in terms of belonging to normally distributed general populations according to the Shapiro–Wilk criterion. The paired t-test was used to determine the reliable differences between the mean values of samplings. The results were considered reliable on condition of the probability value p under 5 % ($p < 0.05$). The validation analysis of data approximation by the linear function (linearization) was performed using Fisher's F-test; determination coefficients (R^2) were at least 0.96 in all cases. The results were presented as the arithmetic mean±standard error of the mean value, n – number of experiments.

RESULTS AND DISCUSSION

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) (Veklich & Kosterin, 2005).

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 95 % as compared to the control) inhibited the activity of Na^+, K^+-ATPase of PM in uterine myocytes (Veklich *et al.*, 2023). At the same time, this substance, used in the same concentration, had virtually no impact on the enzymatic properties of Ca^{2+}, Mg^{2+}-ATPase, "basal" Mg^{2+}-ATPase and Ca^{2+}-ATPase of PM.

Thus, thiacalix[4]arene C-1193 highly effectively ($I_{0.5}$ = 42.1±0.6 nM) inhibited the enzymatic activity of Na^+, K^+-ATPase of PM, without affecting the activities of Mg^{2+}-ATPase, Ca^{2+}-ATPase and Ca^{2+}, Mg^{2+}-ATPase of PM (Veklich *et al.*, 2023). Therefore, for further kinetic interpretation of the impact of thiacalix[4]arene C-1193 on the enzymatic activity of Na^+, K^+-ATPase in PM of the myometrium, we investigated its action on the nature of concentration dependencies of this activity on ATP and Mg ions.

The increase in ATP concentration in the incubation medium in the range from 0.01 to 1 mM led to an increase in the enzymatic activity of Na^+, K^+-ATPase (**Fig. 1**, control) under fixed concentration of $MgCl_2$ (3 mM) in the incubation medium. Hill's method was used to calculate the apparent Michaelis constant K_m for nucleoside triphosphate of ATP and Hill's coefficient n_H which were 195.3±9.7 μM and 1.32±0.12, respectively (n = 5) (**Fig. 2**). The value of K_m in case of ATP for Na^+, K^+-ATPase, obtained by us, correlated with the scientific data. The value of K_m for ATP hydrolysis reaction in the nervous tissue of rats was 260 μM (Veklich & Kosterin, 2005).

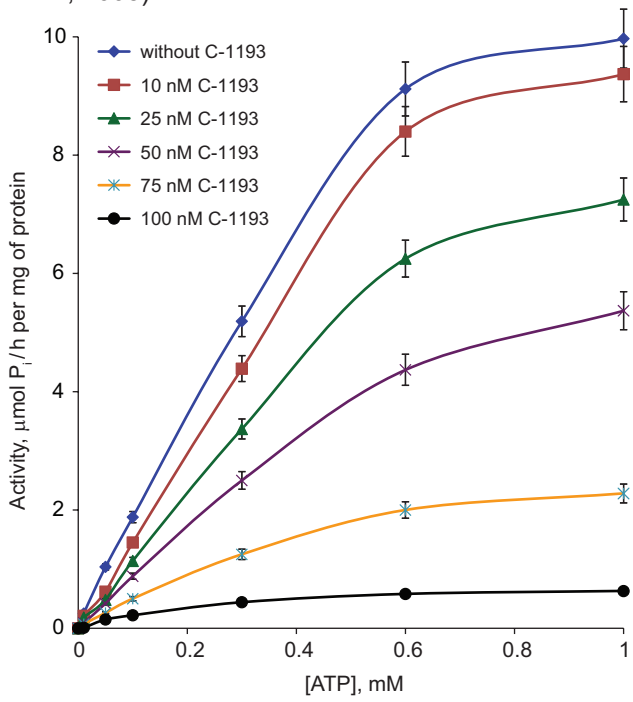


Fig. 1. The impact of different concentrations of thiacalix[4]arene C-1193 on the dependence of the relative enzymatic activity of Na^+, K^+-ATPase in the fraction of plasma membranes of the myometrium cells on the ATP concentration (M±m; n = 5). The concentration of $MgCl_2$ – 3 mM

We studied the impact of thiacalix[4]arene C-1193 on the kinetic parameters, characterizing the affinity of the enzyme to ATP. The action of five concentrations of thiacalix[4]arene C-1193 (10, 25, 50, 75, and 100 nM, respectively) on the concentration dependence of ATP was investigated. In all cases, there was a decrease in the activity of Na^+, K^+-ATPase with a different degree of efficiency, and the dependence of the enzymatic activity on ATP was found to be similar to the relevant control dependence without thiacalix[4]arene C-1193. However, the plateau level of the activity decreased along with the increase in calixarene concentration (**Fig. 1**). The estimated mean values of the apparent Michaelis constant K_m and Hill's coefficients $n_н$ in the presence of different concentrations of thiacalix[4]arene C-1193 did not reliably differ from the control values of the kinetic parameters data in the absence of the effector in the incubation medium (**Fig. 2**). The value of Hill's coefficient $n_н$ demonstrated a positive cooperative effect of the dependence of the enzymatic activity of Na^+, K^+-ATPase on the ATP concentration which almost did not change in the presence of thiacalix[4]arene C-1193 in different concentrations (**Fig. 2**).

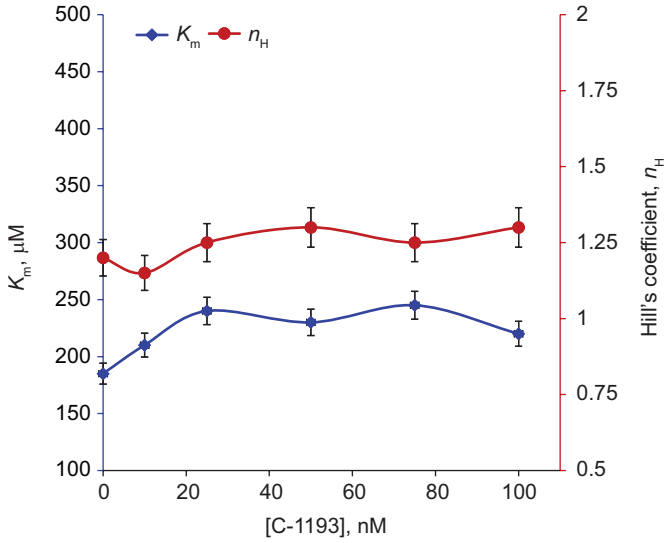


Fig. 2. The action of thiacalix[4]arene C-1193 on the kinetic parameters (Michaelis constant K_m and Hill's coefficient $n_н$) of the ATP effect on the activity of Na^+, K^+-ATPase in the fraction of plasma membranes of the myometrium cells (M±m; n = 5). The concentration of [C-1193] = 0 mM – control. The concentration of $MgCl_2$ – 3 mM

Thus, leading to the inhibition of the activity of Na^+, K^+-ATPase regarding the control, thiacalix[4]arene C-1193 practically did not change the apparent affinity of the enzyme to ATP or the cooperative nature of the enzymatic reaction related to ATP. It is obvious that in this case, the inhibition induced by thiacalix[4]arene C-1193 occurred at the expense of the decrease in the enzyme turnover number, i.e. V_{max} of the ATPase reaction.

We also estimated the kinetic parameters of the action of thiacalix[4]arene C-1193 in the presence of different substrate concentrations of the ATPase reaction (**Fig. 3**, **4**).

It was shown that the application of different ATP concentrations (**Fig. 3**) had a poor effect on the constant K_i and Hill's coefficient $n_н$ for the inhibition induced by thiacalix[4]arene C-1193 (**Fig. 4**).

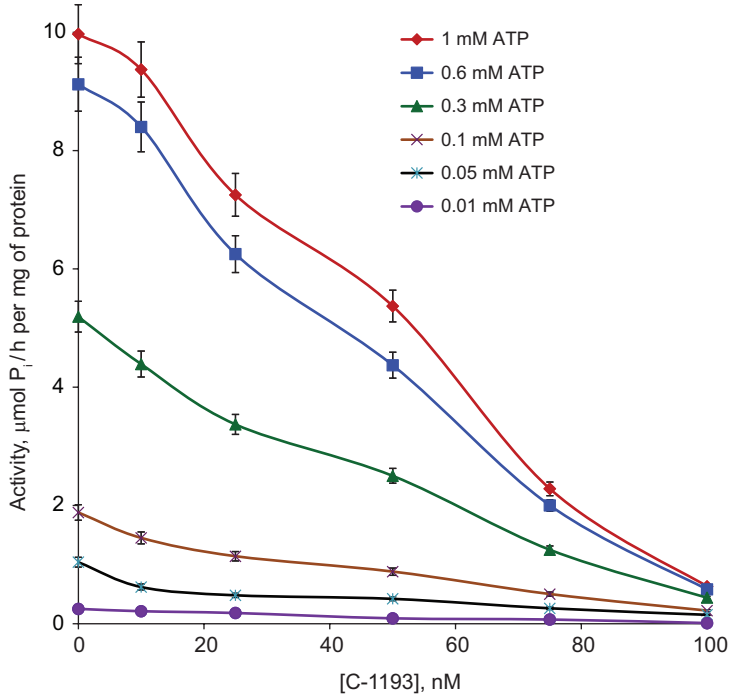


Fig. 3. The action of different concentrations of ATP on the dependence of the relative enzymatic activity of Na^+, K^+-ATPase in the fraction of plasma membranes of the myometrium cells on the concentration of thiacalix[4]arene C-1193 (M±m; n = 5). The concentration of $MgCl_2$ – 3 mM

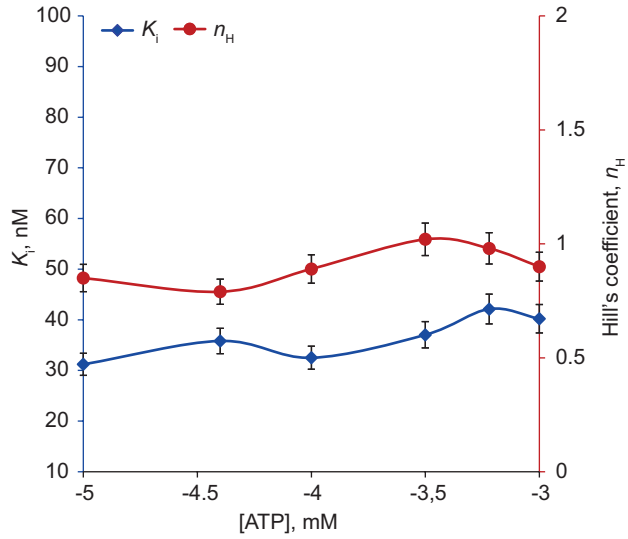


Fig. 4. The action of ATP on the kinetic parameters (inhibition constant K_i and Hill's coefficient n_H) of the effect of thiacalix[4]arene C-1193 on the activity of Na^+, K^+-ATPase in the fraction of plasma membranes of the myometrium cells (M±m; n = 5). The concentration of $MgCl_2$ – 3 mM

Therefore, the inhibiting effect of thiacalix[4]arene C-1193 on the relative enzymatic activity of Na^+, K^+-ATPase did not depend on the amount of ATP in the incubation medium, which demonstrated the absence of competition between ATP and thiacalix[4]arene C-1193. Thus, it may be assumed that the substrate center of Na^+, K^+-ATPase and the hypothetical interaction site for thiacalix[4]arene C-1193 did not overlap on the enzyme surface.

It is known that the relevance of Mg^{2+} for metabolism is relatedattributed to its properties as a promoter of the structure of protein macromolecules, a substrate-binding ion, and an electron transporter. There are many known Mg^{2+}-dependent enzymes, where the role of Mg^{2+} is not limited to substrate activation but is related to the formation of the active (catalytic) center. However, the most widely-known role of Mg^{2+} is its role in the formation of the chelate complex with ATP – the substrate of adenosine triphosphatase reactions. It is believed that Mg^{2+} ions interact with phosphate-charged groups of ATP, polarize them, and raise the reactive ability of the system, facilitating the nucleophilic attack on the terminal phosphate residue of ATP (Bevza *et al.*, 2013).

The enzymatic activity of Na^+, K^+-ATPase in the PM of myometrium increases along with the increase in the concentration of $MgCl_2$ from 0.01 to 3 mM on condition of fixed ATP concentrations (1 mM) in the incubation medium (**Fig. 5**, control (without C-1193)).

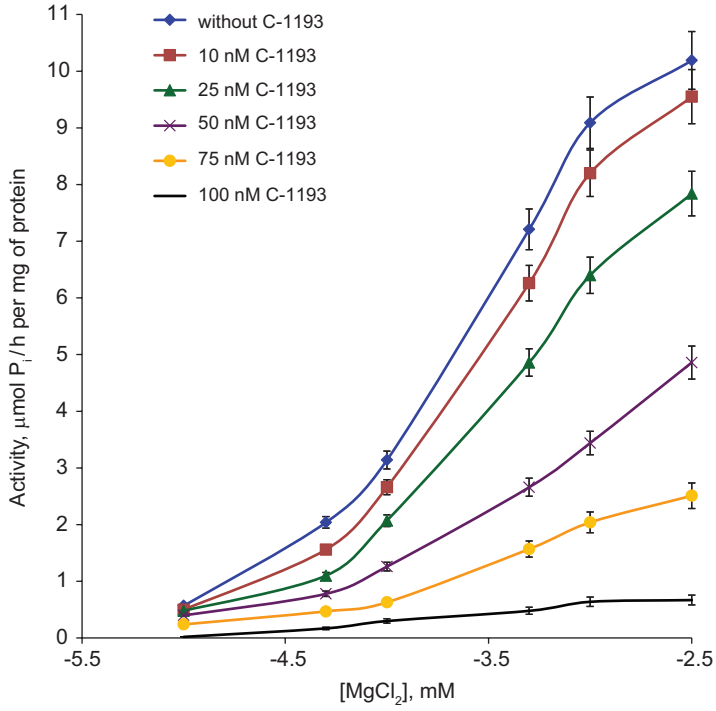


Fig. 5. The action of thiacalix[4]arene C-1193 in different concentrations on the dependence of the relative enzymatic activity of Na^+, K^+-ATPase in the fraction of plasma membranes of the myometrium cells on $MgCl_2$ concentration (M±m; n = 5). The concentration of ATP – 1 mM

The value of the apparent constant of the activation of Na^+, K^+-ATPase K_{Mg} was 164±4.2 µM, the value of Hill's coefficient n_H – 1.05±0.04 (M±m; n = 5) (**Fig. 6**, control).

It should be noted that the concentration of free Mg^{2+} ions in the incubation medium may differ considerably and have no linear dependence on the concentration of $MgCl_2$. Therefore, the presented kinetic parameters were estimated for $MgCl_2$, instead of Mg^{2+}.

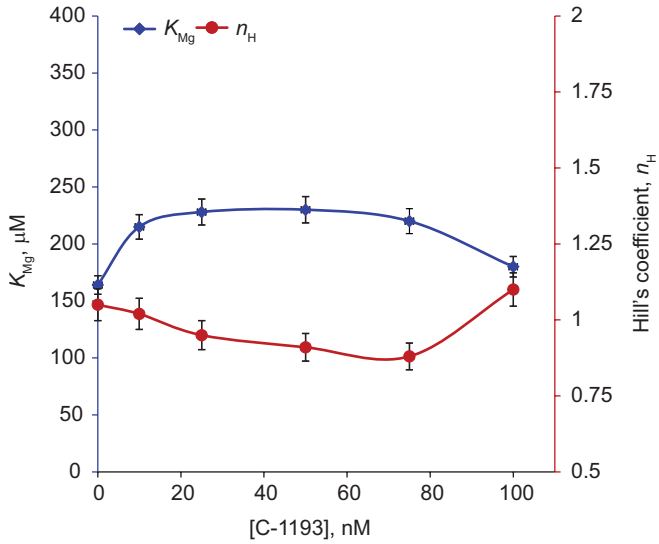


Fig. 6. The action of thiacalix[4]arene C-1193 on the kinetic parameters (the activation constant K_{Mg} and Hill's coefficient n_H) of the impact of $MgCl_2$ on the activity of Na^+, K^+-ATPase in the fraction of plasma membranes of the myometrium cells (M±m; n = 5). The concentration of [C-1193] = 0 mM – control. The concentration of ATP – 1 mM

We studied the impact of thiacalix[4]arene C-1193 on the kinetic parameters of the action of $MgCl_2$. The concentration dependence on $MgCl_2$ was studied in the presence of thiacalix[4]arene C-1193 in different concentrations (**Fig. 5**).

In all cases of introducing thiacalix[4]arene C-1193 into the incubation medium, there was a decrease in the activity of Na^+, K^+-ATPase with a different degree of efficiency. It was demonstrated that the dependence of the constant of the activation by magnesium chloride on the concentration of thiacalix[4]arene C-1193 increased insignificantly to 220±32 μM with an increase in the concentration of thiacalix[4]arene C-1193 to 75 nM. In the case of a further increase in the concentration of thiacalix[4]arene, K_{Mg} decreased almost down to the control level (under the concentration of thiacalix[4]arene C-1193 of 100 nM (**Fig. 6**)). At the same time, the value of Hill's coefficient n_H practically did not change in the presence of thiacalix[4]arene C-1193 in different concentrations (n_H = 1.05±0.04 − 1.10±0.05).

The study has shown that the application of different $MgCl_2$ concentrations (**Fig. 7**) did not have a considerable impact on the inhibition constant K_i and Hill's coefficient regarding thiacalix[4]arene C-1193 (**Fig. 8**). Thus, the inhibitory action of thiacalix[4] arene C-1193 on the relative enzymatic activity of Na^+, K^+-ATPase did not depend on the number of Mg ions in the incubation medium, indicating the absence of competition between Mg^{2+} and thiacalix[4]arene C-1193.

Thus, the obtained results demonstrated that the highly efficient inhibitory action of thiacalix[4]arene C-1193 on Na^+, K^+-ATPase was uncompetitive regarding ATP and Mg ions and related to the decrease in the enzyme turnover number in its presence.

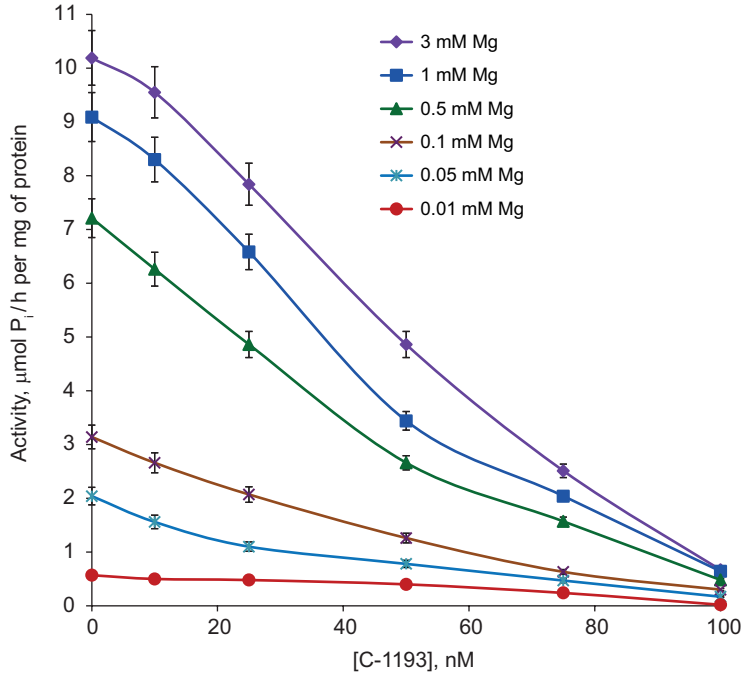


Fig. 7. The action of different concentrations of $MgCl_2$ on the dependence of the relative enzymatic activity of Na^+, K^+-ATPase in the fraction of plasma membranes of the myometrium cells on the concentration of thiacalix[4]arene C-1193 (M±m; n = 5). The concentration of ATP – 1 mM

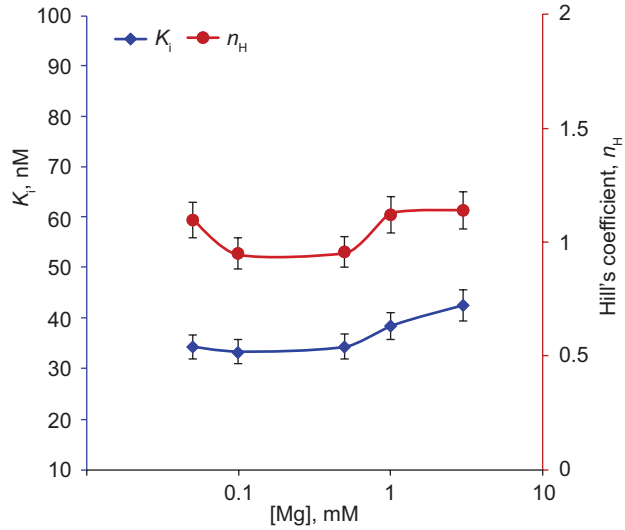


Fig. 8. The action of $MgCl_2$ on the kinetic parameters (inhibition constant K_i and Hill's coefficient n_H) of the impact of thiacalix[4]arene C-1193 on the activity of Na^+, K^+-ATPase in the fraction of plasma membranes of the myometrium cells (M±m; n = 5). The concentration of ATP – 1 mM

It was previously shown (Veklich *et al.*, 2023) that a highly selective inhibitor Na^+, K^+-ATPase of the plasma membrane, calix[4]arene C-1193, inhibited the amplitude of

spontaneous isometric contractions depending on the dose, and modulated their kinetics, including a decrease in force (F_{max}, F_C and F_R), velocity (V_C and V_R) and impulse (I_C, I_R and I_0) parameters of some spontaneous contraction-relaxation cycles accompanied by an increase in the frequency of motility. However, under *in vivo* conditions, the functioning of muscle tissues takes place in the isotonic or auxotonic modes (Oslin *et al.*, 2022). Thus, our study aimed at investigating possible effects and specification of the mechanisms of the action of calix[4]arene C-1193 on the mechanokinetics of smooth muscles of the uterus of rats under the isotonic registration mode, in conditions, maximally approximating the physiological ones.

It was found that contrary to the isometric mode of registering the contractile activity, under isotonic conditions, calix[4]arene C-1193 caused a reliable increase in spontaneous contractions of the myometrium preparations (**Fig. 9**). For instance, due to the effect of this compound in the concentration of 10 μM, there was an increase in the amplitude of contractions up to 129.0±6.8 % on average (n = 5, p <0.05). Noteworthy, under the action of C-1193, the frequency of spontaneous contractions tended to decrease.

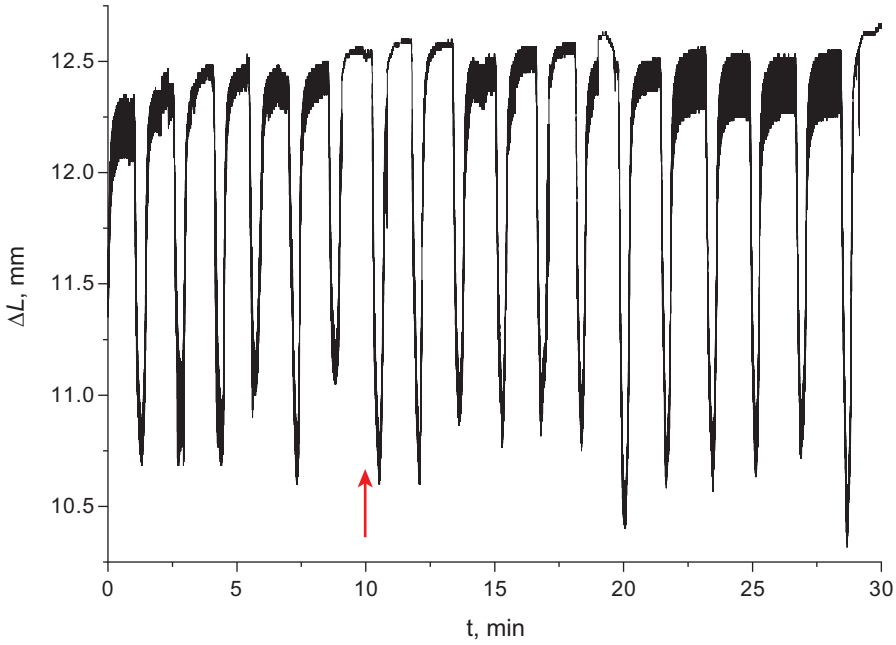


Fig. 9. The typical mechanogram of the spontaneous isotonic contractions of the longitudinal smooth muscles in uterine horns of rats in control and under the effect of thiacalix[4]arene C-1193 (10 μM, the moment of addition is marked with an arrow)

The method of complex mechanokinetic analysis was further applied to specific contraction-relaxation cycles (in control and under the effect of C-1193) (Kosterin & Tsymbalyuk, 2023). It was found that C-1193 induced an increase in the contraction parameters: maximal contraction (ΔL_{max}) and contractions at the inflexion points ΔL_C and ΔL_R) (**Fig. 10*A***). For instance, under the effect of C-1193, the average parameters of ΔL_{max}, ΔL_C and ΔL_R were, respectively, 129.0±2.8 %, 132.8±2.3 %, and 128.8±3.0 % (in all cases n = 5, p <0.05).

In these conditions, the indices of temporal parameters of the amplitude (τ_{max}) and the characteristic time of contraction and relaxation (τ_C and τ_R) remained at the control level, amounting to the following values, respectively: 88.1±4.0 %, 92.5±5.4 % and 91.5±4.1 % (in all cases n = 5, p >0.05) (**Fig. 10*B***).

Under the action of calix[4]arene C-1193, the greatest changes were noted for the velocity parameters of the phases of contraction (V_C) and relaxation (V_R) (**Fig. 10*C***). For instance, against 10 μM of this compound, the parameters V_C and V_R were, on average, 143.5±3.1 % and 155.6±2.9 % compared to the control, respectively (in both cases, n = 5, p <0.01). Importantly, the changes in both parameters of maximal velocities were completely (down to statistically negligible) eliminated by their normalization in terms of the contraction amplitude (**Fig. 10*D***).

We can assume the reasons for the changes in the myometrium motility under the effect of calix[4]arene C-1193. The first thing to be noted is the increase in the amplitude of spontaneous contractions under the impact of the compound without any considerable changes in their frequency. The effects of increasing the contractile function of the muscles are generally notable for blocking Na^+, K^+-ATPase of the PM and may be related to the transfer of the activity of Na^+/Ca^{2+}-exchanger (determined by the gradient of Na^+ ions in the myoplasm) into the reverse mode (Kim *et al.*, 2011; Rodrigues *et al.*, 2022).

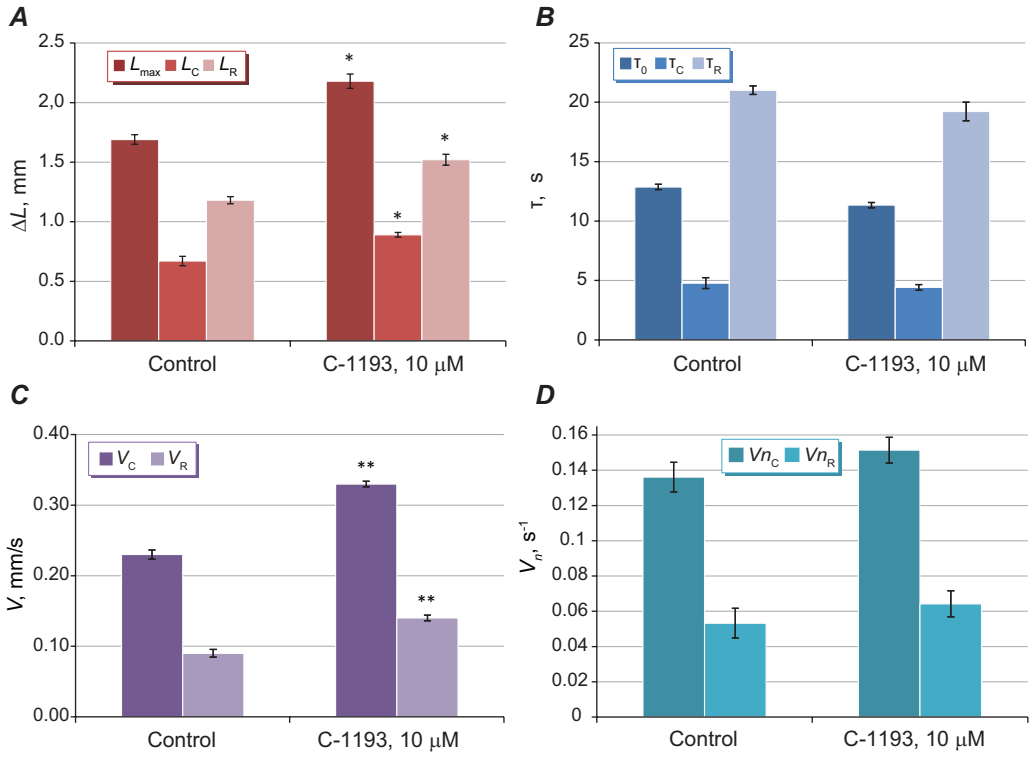


Fig. 10. The parameters of the spontaneous isotonic contractions of rat myometrium in the control and under the action of calix[4]arene C-1193 (10 μM): ***A*** – temporal parameters (τ_0, τ_C and τ_R); ***B*** – lenth parameters (ΔL_{max}, ΔL_C and ΔL_R); ***C*** – velocity parameters (V_C and V_R); ***D*** – normalized velocities of the phases of contraction and relaxation (V_{nC} and V_{nR}). n = 5; * – p <0.05, ** – p <0.01 – the difference is reliable as compared to the control

Our study also demonstrated that in addition to the amplitude, the other block of mechanokinetic parameters that changed considerably at the action of C-1193 was velocity parameters (V_C and V_R) (**Fig. 10*C***). The velocity of the process of myometrium contraction is mostly conditioned by the kinetics of the uptake of Ca^{2+} ions to the myoplasm (Cherepanov *et al.*, 2023). In contrast, during the relaxation phase, the contribution is mostly made by various processes of extrusion of these cations from the cytoplasm (via transporting into the extracellular medium through the plasma membrane using Ca^{2+}-pump and Na^+/Ca^{2+}-exchanger, as well as sequestration in the intracellular depots – sarcoplasmic reticulum and mitochondria) (Wray, 2015; Cherepanov *et al.*, 2023). It should be noted that under the action of C-1193, both velocities V_C and V_R increased in a similar way, and their normalization regarding the amplitude of spontaneous contractions caused the loss of statistically significant differences regarding the corresponding normalized velocities in the control (**Fig. 10*D***). Similar effects regarding the normalized maximal velocities of the contraction and relaxation phases were obtained by us for the modulation of isometric spontaneous contractions by calix[4]arene C-1993. Thus, our results suggest that under these conditions, the change in the velocity parameters may not be specific regarding some Ca^{2+}-transporting systems of myocytes.

Since the ability of muscles to ensure active contractions is extremely important, we estimated the changes in the energy parameters of spontaneous contractions under the effect of calix[4]arene C-1193. It was found that under these conditions, there was a considerable increase in the maximal work of the muscle A_{max} (on average up to 128.9±3.3 % compared to the control, accepted as 100 %, $p < 0.01$, $n = 5$), and the parameter of the work, done by the muscle with the maximal velocity of the contraction A_{tc} (on average 133.4±5.4 % compared to the control, $p < 0.05$, $n = 5$) (**Fig. 11*A***).

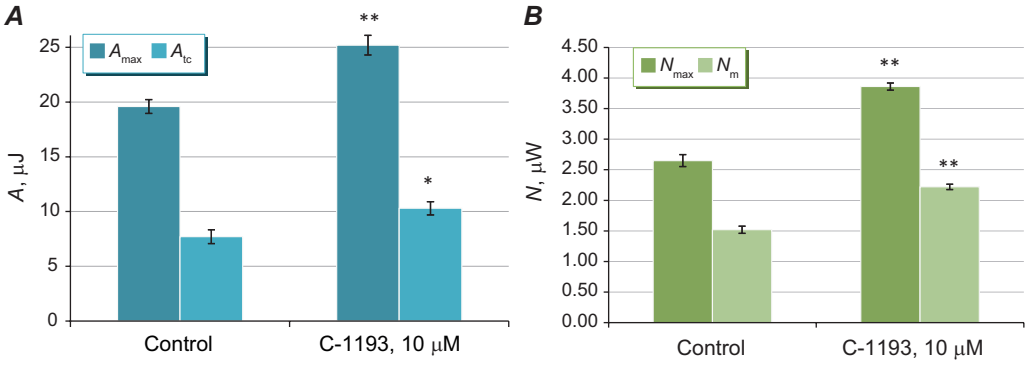


Fig. 11. The energy parameters of spontaneous isotonic contractions-relaxations of the myometrium of rats in control and at the action of calix[4]arene C-1193 (10 μM): ***A*** – work (A_{max} – maximal work and A_{tc} – index of the work, done by the muscle during the contraction in the point τ_C); ***B*** – intensity (N_{max} – maximal power of the muscle, N_m – average power of the muscle). The data are presented as M±m ($n = 5$, * – $p < 0.05$, ** – $p < 0.01$ – the difference is reliable regarding the control)

Some changes in the rat myometrium intensity were also induced in the presence of C-1193 (**Fig. 11*B***): the parameter of the maximal power of the muscle N_{max} was, on average, 145.6±1.6 % compared to the control, accepted as 100 % ($p < 0.01$, $n = 5$), and the average power of the muscle N_m was at the level of 146.0±2.0 % ($p < 0.01$, $n = 5$).

Then, to investigate the ability of C-1193 to impact the processes of the excitation-contraction coupling at the activation of the depolarization-activated current of Ca^{2+} ions

via the sarcolemma of myocytes, we studied the contractions of smooth muscle preparations, activated by the application of the high-potassium solution (HPS, 80 mM).

It was found that the preliminary incubation (for 30 min) of the muscles with this calix[4]arene was accompanied by an increase in the amplitude of the phase (on average to 115.1±3.4 %, n = 5, p <0.05) and tonic (on average to 122.4±3.7 %, n = 5, p <0.05) components of the HPS-activated contraction (**Fig. 12*A*** and **13**).

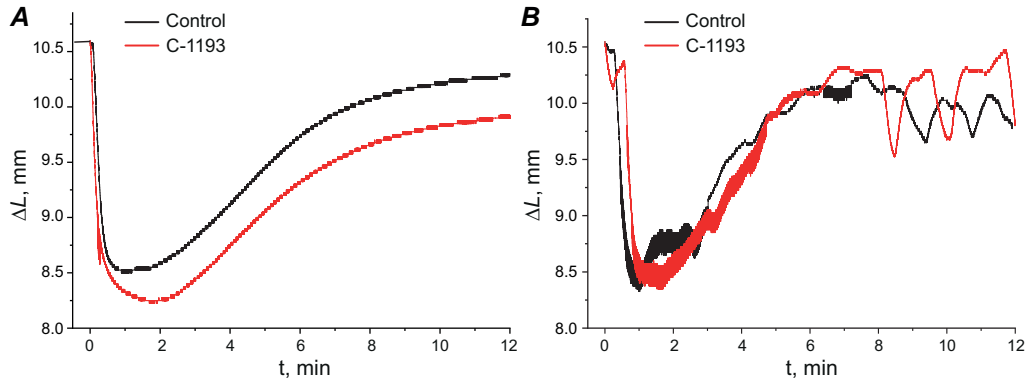


Fig. 12. The typical mechanograms of the isotonic contractions of the longitudinal smooth muscles in uterine horns of rats in control and under the effect of calix[4]arene C-1193 (100 μM), activated by the application of the high-potassium (80 mM) solution (***A***) and oxytocin (0.1 IU) (***B***)

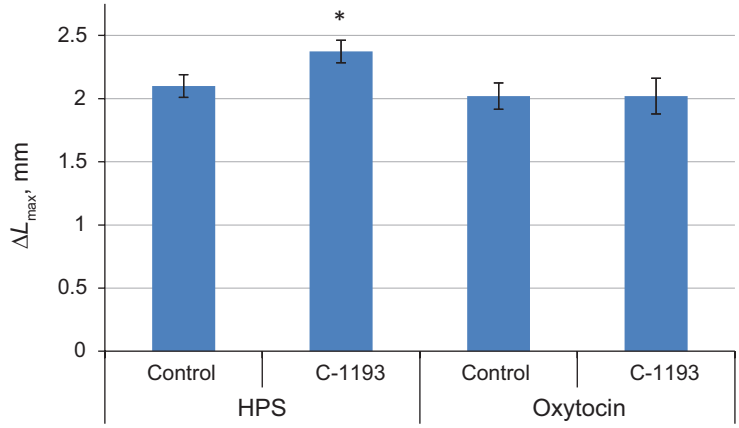


Fig. 13. The amplitude of the phase components of the isotonic myometrium contractions of rats in control and under the action of calix[4]arene C-1193 (10 μM), induced by the application of the high-potassium solution (HPS, 80 mM) and oxytocin (0.1 IU). n = 5; * – p <0.05 – the difference is reliable as compared to the control

The mechanokinetic analysis, conducted by the method of Kosterin and Burdyga (Burdyga & Kosterin 1991), demonstrated that under these conditions, there were no differences in both parameters of the normalized maximal velocities of the phases of contraction (V_{nC}) and relaxation (V_{nR}), which, on average, were 102.2±7.5 % and 89.5±7.6 %, respectively (in both cases n = 5, p >0.05) (**Fig. 14**).

The absence of differences in the ratio of the phases of the phase and tonic components under the action of C-1193 and the changes in the kinetics of the contraction-relaxation processes in case of HPS-induced contractions, may also indicate the absence of

some selective targets among Ca^{2+}-transporting systems, used in this type of contraction: the energy-independent uptake of these cations via voltage-gated Ca^{2+}-channels (Garrett *et al.*, 2022) and energy-dependent extrusion of these cations from myocytes (Wray, 2015; Cherepanov *et al.*, 2023).

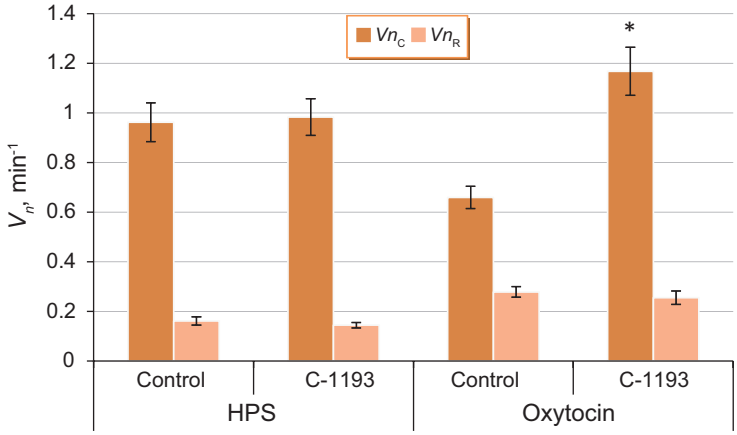


Fig. 14. The normalized maximal velocities of the phases of contraction and relaxation (V_{nC} and V_{nR}) of the isotonic contractions of the rat myometrium in control and under the effect of calix[4]arene C-1193 (10 μM), induced by the application of the high-potassium solution (HPS, 80 mM) and oxytocin (0.1 IU). n = 5; * – p <0.05 – the difference is reliable as compared to the control

A uterotonic hormone, oxytocin, is a relevant factor in regulating the contractile activity of the myometrium. Therefore, in the next stage, we investigated the mechanokinetic effects of C-1193 on the myometrium contractions, activated by this substance.

It was found that calix[4]arene C-1193 did not change the force of the phase and tonic components of the oxytocin-induced contractions of the myometrium (**Fig. 12*B*** and **13**). The estimation of the normalized maximal velocities of the phases of contraction and relaxation of these mechanograms showed that in the presence of the substance in the solution, washing the smooth muscle preparations, there was an increase in V_{nC}, whereas the parameter V_{nR} remained at the control level (**Fig. 14)**.

CONCLUSIONS

The results of our studies indicate that thiacalix[4]arene C-1193 has a non-competitive mechanism of inhibition of the activity of Na^+, K^+-ATPase and does not have a specific effect on the Ca^{2+}-transport systems of uterine myocytes. Judging by the effects of this compound on the contraction of multicellular muscle preparations and the registration of Ca^{2+} signal in rat uterine myocytes, it does not have a significant influence on other (except those caused by blocking the sodium pump of the plasma membrane) cellular mechanisms of regulation of excitation-contraction processes.

The experimental data obtained using thiacalix[4]arene C-1193, an inhibitor of Na^+,K^+-ATPase, may be of great importance for elucidating the membrane mechanisms of cation exchange in smooth muscles, in particular, when studying the role of the plasma membrane in providing electro- and pharmacomechanical coupling in them, as well as in the regulation of ionic homeostasis in myocytes. Notably, this thiacalix[4]arene can be considered as a promising compound for the development of drugs with a mild uterotonic effect.

ACKNOWLEDGMENTS

The study was conducted with the financial support of the state-registered grants Nos. 0123U100894, 0124U000224.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest: the authors declare that they have no conflict of interest.

Human Rights: this article does not contain any studies with human subjects performed by any of the authors.

Animal Studies: all international, national and institutional guidelines for the care and use of laboratory animals were followed.

AUTHOR CONTRIBUTIONS

Conceptualization, [S.K.; O.T.; T.V.; V.K.; S.C.]; methodology, [O.T.; T.V.; S.C.; O.M.]; validation, [O.T.; T.V.; S.C.]; formal analysis, [O.T.; T.V.; O.M.].; investigation, [O.T.; T.V.; O.M.; S.C.]; resources, [O.T.; T.V.; S.C.; S.K.]; data curation, [O.T.; T.V.; S.C.; S.K.; V.K.]; writing – original draft preparation, [O.T.; T.V.; O.M.; S.C.; S.K.; V.K.]; writing – review and editing, [O.T.; T.V.; O.M.; S.C.; V.K.; S.K.]; visualization, [O.T.; T.V.; O.M.; S.C.]; supervision, [O.T.; T.V.; V.K.; S.K.]; project administration [S.K.; V.K.]; funding acquisition, [-].

All authors have read and agreed to the published version of the manuscript.

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КІНЕТИЧНІ ЗАКОНОМІРНОСТІ ДІЇ ТІАКАЛІКС[4]АРЕНУ С-1193 НА Na^+,K^+-АТФ-азну АКТИВНІСТЬ ПЛАЗМАТИЧНОЇ МЕМБРАНИ ТА СКОРОТЛИВУ АКТИВНІСТЬ МІОМЕТРІЯ

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Обґрунтування. Na^+,K^+-АТФ-аза (натрієва помпа) – електрогенна Ca^{2+}-незалежна Mg^{2+},Na^+,K^+-АТФ-залежна транспортна система плазматичної мембрани, яка здійснює активне перенесення одновалентних іонів Na і K, тим самим підтримуючи їхні електрохімічні градієнти, які є необхідними для нормального функціонування клітини. У разі багатьох поширених патологій відбувається порушення актив-

ності натрієвої помпи. Тому пошук оборотних ефекторів – селективних інгібіторів і активаторів, які були би здатні спрямовано впливати на Na^+,K^+-АТФ-азу, відновлюючи її активність за патологічних станів, є вельми перспективним. Метою роботи було дослідити вплив тіакалікс[4]арену С-1193 на залежність Na^+,K^+-АТФ-азної активності плазматичної мембрани клітин міометрія від концентрації АТФ та іонів Mg, а також на ізотонічну скорочувальну активність міометрія.

Матеріали та методи. В експериментах, виконаних на суспензії перфорованих плазматичних мембран клітин міометрія, досліджували інгібувальну дію тіакалікс[4]арену С-1193 (25,27-дибутокситіакалікс[4]арен-біс-гідроксиметилфосфонова кислота) на кінетичні характеристики Na^+, K^+-АТФ-азної активності. Реєстрацію скорочувальної активності гладеньком'язових смужок поздовжніх гладеньких м'язів рогів матки здійснювали тензометричним методом в ізотонічному режимі. Вивчення скоротливої активності м'язових препаратів здійснювали методами механокінетичного аналізу.

Результати. Тіакалікс[4]арен С-1193, інгібуючи Na^+, K^+-АТФ-азу, не змінює кінетичні параметри (K_m, n_H) залежності швидкості реакції від концентрації субстрату. На спорідненість Na^+, K^+-АТФ-ази до АТФ калікс[4]арен С-1193 практично не впливає, що свідчить про відсутність конкуренції між центрами зв'язування АТФ та С-1193. Так само можна відзначити брак впливу на спорідненість і кооперативний ефект іонів Mg. В обох випадках спостерігали суттєве зменшення максимальної швидкості гідролізу АТФ, що у поєднанні з відсутністю впливу на константи спорідненості свідчить про неконкурентний механізм інгібуванням сполукою С-1193 Na^+, K^+-АТФ-азної активності.

Виявлено, що тіакалікс[4]арен С-1193 (у концентрації 10 μM) модулює ізотонічні реакції багатоклітинних препаратів міометрія, активовані гіперкалієвою деполяризацією і утеротонічним гормоном окситоцином. Також встановлено, що за дії С-1193 збільшується амплітуда скорочення і зростають механокінетичні параметри: скорочення (ΔL_{max}, ΔL_C та ΔL_R) і швидкостей (V_C і V_R). Нормування максимальних швидкостей фаз скорочення та розслаблення (V_C і V_R) на амплітуду скорочень призводить до втрати статистично значущих відмінностей між цими параметрами у контролі та за дії тіакалікс[4]арену.

Висновки. Результати наших досліджень доводять, що тіакалікс[4]арен С-1193 має неконкурентний механізм пригнічення активності Na^+, K^+-АТФ-ази, а також не має специфічної дії на Ca^{2+}-транспортні системи міоцитів матки.

***Ключові слова**:* міометрій, Na^+,K^+-АТФ-аза, плазматична мембрана, тіакалікс[4]арен С-1193, гладеньком'язові клітини, кінетичні властивості АТФ-ази, скорочення, механокінетичні параметри

Received / Одержано 24 January, 2025 | Revision / Доопрацьовано 13 February, 2025 | Accepted / Прийнято 19 March, 2025 | Published / Опубліковано 26 March, 2025