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CALIX[4]ARENE C-956 AS A SELECTIVE INHIBITOR OF Ca^{2+}-PUMP OF THE PLASMA MEMBRANE AND A MODULATOR OF THE CONTRACTILE FUNCTION IN THE MYOMETRIUM

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Background. At present, creating and testing pharmacological instruments for selective inhibition of Ca^{2+}-pump of the plasma membrane, which would become the foundation for medical preparations, for instance, for the treatment of the impaired excitability of the cardiac and smooth muscles, remains critically significant. We have demonstrated in our previous experiments that calix[4]arene C-956 is effective in inhibiting Ca^{2+}, Mg^{2+}-ATPase activity of the plasma membrane of myometrium cells. The aim of this study was to investigate the regularities and mechanisms of the impact of calix[4] arene C-956 on Ca^{2+}-transporting activity of Ca^{2+}, Mg^{2+}-ATPase of the plasma membrane (PM) and the contractile function of rat myometrium.

Materials and Methods. The experiments were conducted using outbred white non-pregnant rats. Ca^{2+}-transporting activity of myocytes PM preparations loaded with Ca^{2+}-sensitive fluorescent probe fluo-4 AM was investigated. The registration of the contractile activity in the preparations of longitudinal smooth muscles of uterine horns with preserved endothelium was done in the isometric mode.

Results. It was determined that calix[4]arene C-956 causes blocking of the transport function of the calcium pump of preparations of plasma membranes of uterine myocytes. The C-956 compound causes an increase in the amplitude of spontaneous contractions and a change in their mechanokinetic parameters during a short-term effect on multicellular preparations of rat myometrium. Calix[4]arene C-956 also significantly

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affects the contractions caused by high-potassium depolarization of the PM and oxytocin, increasing their amplitude and decreasing the rate of relaxation. Blocking the synthesis of nitric oxide significantly enhances the effects of C-956 on spontaneous and high-potassium- and oxytocin-induced contractions of the myometrium.

Conclusions. The results of our research indicate that the main target of the action of calix[4]arene C-956 on myocytes is the calcium pump of the PM. With the preliminary inhibition of nitric oxide synthases followed by the use of C-956, we were able to fully demonstrate the contribution of the calcium pump of the PM to the regulation of uterine contractions.

Keywords: myometrium, Ca^{2+}-pump of the plasma membrane, calix[4]arene C-956, contractions, mechanokinetic parameters, Ca^{2+}-signal

INTRODUCTION

Ca^{2+} ions play an important role in the maintenance and regulation of cell functions, serving as a secondary messenger and extracellular regulator. An especially critical role is attributed to the intracellular concentration of Ca^{2+} in excitable tissues, including visceral smooth muscles (Dirksen *et al*., 2022). After the excitation of the smooth muscle cell, accompanied with the increase in the cytosolic Ca^{2+} ($[Ca^{2+}]_i$), an obligatory prerequisite for the normal functioning of a myocyte is a fast return of this cation concentration to the initial level. The maintenance of $[Ca^{2+}]_i$ on the basal level is ensured by the energy-dependent transporters of the plasma membrane (PM) and intracellular calcium depots (sarcoplasmic reticulum and mitochondria). The systems of Ca^{2+} extrusion into the extracellular space are Na^+/Ca^{2+}-exchanger and Ca^{2+}-pump of the plasma membrane (PMCA), where the latter plays the role of the highly affine system of Ca^{2+} release, ensuring long-term maintenance of the basal concentration of these cations in the dormant state (Liu *et al*., 2007; Krebs, 2022; Adasme *et al*., 2023).

Basically, four isoforms of PMCA (PMCA1-4) are expressed in mammals. It is noteworthy that PMCA1 and PMCA4 are non-selectively expressed by all cells: PMCA1 is a “housekeeping” isoform, and PMCA4 is notable for the prevailing majority of cell types (Wu *et al*., 2018; Adasme *at al*., 2023; Naffa *et al*., 2024). All primary PMCA transcripts are subject to alternative splicing, due to which some molecules of the pumps have a conservative PDZ-domain (specialized protein interaction domain, which is named after the first letters of the names of three proteins: the postsynaptic density-95, discs-large and zona occludens-1) in the intracellular C-terminal part of the molecule. For example, PMCA isoforms 1 and 4 are expressed in the uterine tissues, where PDZ-containing molecules dominate (Sluysmans *et al*., 2022). It ensures the interaction between a PMCA molecule and important cytosol proteins, including constitutive nitrogen oxide synthases (NOS) (Schuh *et al*., 2001; Williams *et al*., 2006; Mohamed *at al*., 2009). PMCA maintains a low basal concentration of $[Ca^{2+}]_i$ near NOS, causing them to be in a mostly inhibited state (Duan *at al*., 2013; Zhang *et al*., 2014).

At present, the task of creating and testing pharmacological instruments for selective inhibition of PMCA, which would become the foundation for medical preparations, for instance, for the treatment of the impaired excitability of the cardiac and smooth muscles, remains critically significant. It has been proven in our previous studies that some macrocyclic compounds, calix[4]arenes (including C-90, C-716, C-960, C-956, C-975, and C-1087), are selective inhibitors of this enzyme and can perform this function when

applied to such model systems of different structural levels as the preparations of the suspensions of myocyte PM and pluricellular preparations of visceral smooth muscles (Veklich *et al.*, 2016, 2023; Tsymbalyuk, 2021; Tsymbalyuk *et al.*, 2023).

We have demonstrated in our previous experiments that calix[4]arene C-956 is effective in inhibiting Ca^{2+}, Mg^{2+}-ATPase activity of the PM of myometrium cells, the value of the inhibition coefficient $I_{0.5}$ is 15.0±0.5 μM, the value of Hill coefficient n_H is 0.55±0.01 (Veklich *et al.*, 2018). We have assumed that if calix[4]arene C-956 inhibits the activity of Ca^{2+}-transporting Ca^{2+}, Mg^{2+}-ATPase of PM, it could be expected that under the effect of this compound, Mg^{2+}, ATP-dependent transportation of Ca ions via PM would be inhibited and thus the concentration of these ions in the myoplasm would increase, changing the contractile activity of myocytes. Further studies have been targeted at resolving these issues. Hence, the aim of this study was to investigate the regularities and mechanisms of the impact of calix[4]arene C-956 on Ca^{2+}-transporting activity of Ca^{2+}, Mg^{2+}-ATPase of the PM and the contractile function of rat myometrium.

MATERIALS AND METHODS

The experiments were conducted using outbred white female rats (the average weight of animals was 200–250 g). All the manipulations with animals were conducted according to the International Convention for the Protection of Animals and the Law of Ukraine “On Protection of Animals from Cruelty” (the Minutes of the meeting of bioethics commission of SSC Institute of Biology and Medicine No. 3 dated May 2, 2019). The animals were killed by the displacement of cervical vertebrae after sedation with ether.

Calix[4]arene C-956 (5, 11, 17, 23-tetra(trifluoro)methyl-(phenyl-sulfonylimino) methylamino-25, 27-dioctyloxy-26,28-dipropoxycalix[4]arene) (**Fig. 1**) was synthesized and characterized using NMR and infrared spectroscopy methods at the Department of the Chemistry of Macrocyclic Compounds at the Institute of Organic Chemistry, the NAS of Ukraine (the department is headed by the full member of the NASU, Prof. V. I. Kalchenko).

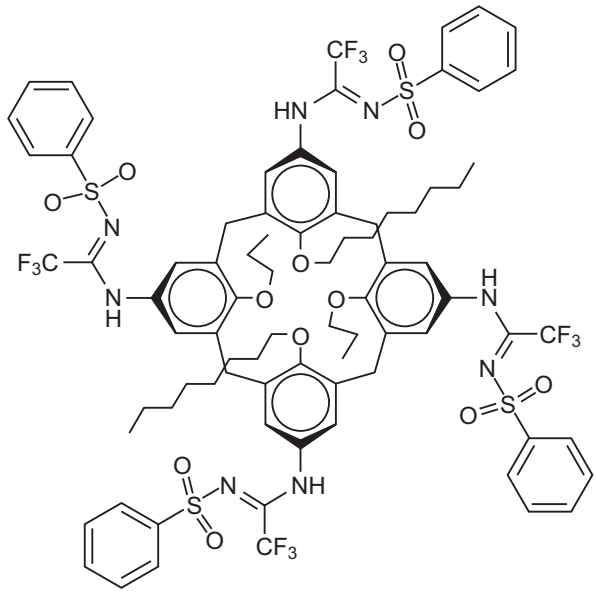

Fig. 1. The structural formula of calix[4]arene C-956

The study of Ca^{2+}-transporting activity of the preparations of myocyte PMs. To investigate the transporting activity of Ca^{2+}, Mg^{2+}-ATPase of PM, we used a Ca^{2+}-sensitive fluorescent probe, fluo-4 AM (2 μM), previously used to load PM vesicles. The PM fraction of uterine SM cells was isolated from the porcine myometrium, as described before (Veklich & Kosterin, 2005).

The accurate determination of the transporting activity of the PM Ca^{2+}-pump requires the knowledge of the topology of membrane fragments of the PM fraction. According to the data reported by T. Veklich & S. Kosterin (2005), the applied method of obtaining PM vesicles is characterized by the availability of a prevailing majority of the fragments locked with the cytoplasm outside ("inside out"), which is confirmed by the ATP-dependent accumulation of $^{45}Ca^{2+}$, inhibited by oxytocin only after its previous loading into the vesicles. Using the determination of the relative activity of the PM marker enzymes (Na^+, K^+-ATPase, and "basal" Mg^{2+}-ATPase), we found that the PM fraction consisted of approximately 50 % vesicles, locked "inside out", the other half being composed of unlocked fragments and the ones, locked "outside out". Thus, vesicle membrane fragments are a convenient instrument to study the systems of active Ca^{2+} transportation, ensuring the transfer of the cation from the myoplasm into the extracellular space.

The transporting activity of Ca^{2+}, Mg^{2+}-ATPase of PM was initiated by introducing 2 μM $CaCl_2$ after the stabilization of the fluorescent response of the probe (for about 5–10 min). The administration of Ca^{2+} into the incubation medium led to a gradual increase in the quantum efficiency of the probe (**Fig. 2**). A similar increase in the quantum efficiency was characterized by the absence of sensitivity to 100 nM thapsigargin (a specific inhibitor of Ca^{2+}, Mg^{2+}-ATPase of sarcoplasmic reticulum (Darbost *et al.*, 2005)), 1 mM of sodium azide (the inhibitor of the mitochondrial breathing (Di Leva *et al*., 2008)), but completely inhibited by the introduction of eosin Y (the data are not presented) (non-specific inhibitor of PM ATPases (Gatto *et al.*, 1995)). If Mg^{2+} and ATP were unavailable in the incubation medium, there was practically no observable increase in the fluorescent response (**Fig. 2**). The introduction of the aliquot of EDTA solution (2 mM) into the incubation medium along with the Ca^{2+} ionophore A-23187 (2.5 μM) decreased the fluorescent signal of fluo-4 and led to the termination of the further increase in the quantum efficiency of the probe (**Fig**. **2**, 450 s).

According to the obtained results, the system, suggested by us, allows the registration of Ca^{2+} transportation into PM vesicles, the main component of which is conditioned by the activity of Ca^{2+}-pump in PM.

The study on the contractile activity of myometrium strips. The contractile activity of the rat uterine horns was investigated by tenzometric methods in the isometric mode using the preparations of the longitudinal smooth muscles (2×10 mm) with the intact endothelium; the study of the spontaneous contractions involved the use of the fragments of uterine horns from the ovarian section.

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

The preparations of smooth muscles were placed into the working chamber of tenzometric equipment with the flowing Krebs solution (the flow rate of 5 mL/min and thermostating at 37.5±0.3 °C) and left for 1 h until the occurrence of spontaneous contractions with constant amplitude and frequency.

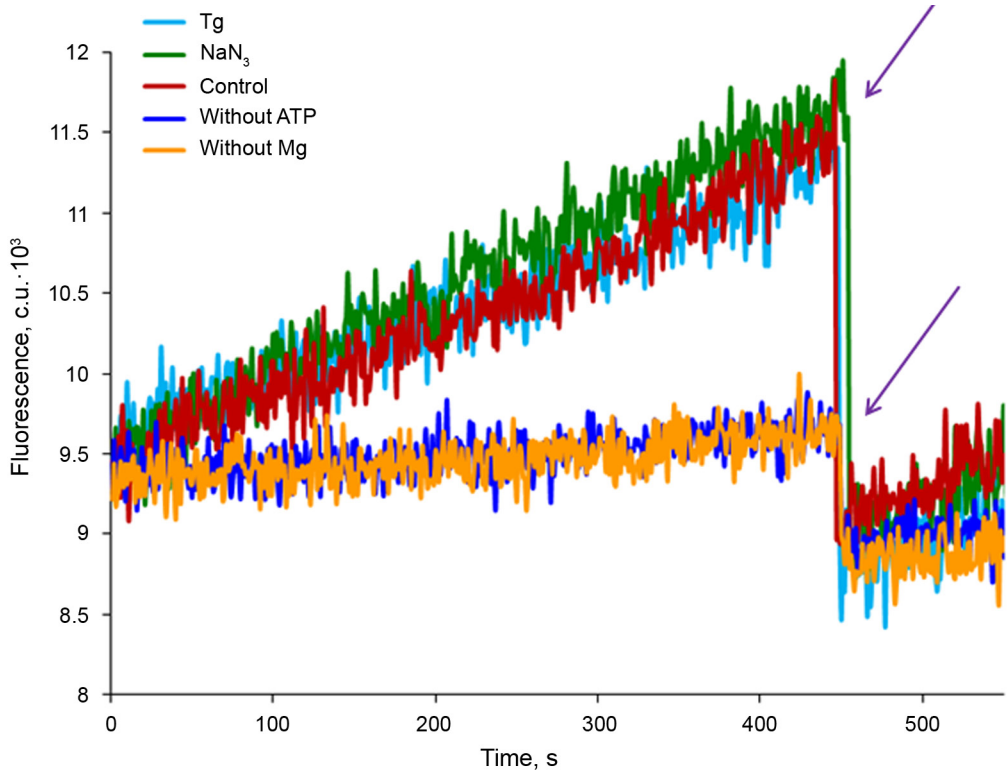

Fig. 2. The kinetics of the fluorescence of Ca^{2+}-sensitive probe fluo-4 under the simulation of Mg^{2+}-ATP-dependent accumulation of Ca^{2+} ions in the vesicles ("inside out") of PM of myometrium cells. The asterisks indicate the moment of introducing the aliquot of the calcium ionophore A-23187 solution along with EDTA (final concentrations in the incubation medium were 5 μM and 2 mM, respectively). The results of the typical experiment are presented

Calix[4]arene C-956 was preliminarily dissolved in DMSO and administered to the solutions in the concentration of 10^{-5} M (DMSO concentration was 0.1 %); the control contractions were registered against the background of 0.1 % DMSO.

The high-potassium solution (80 mM) was prepared by the isotonic replacement of Na^+ ions with K^+ ions. The study also involved the use of the solution of the uterotonic hormone of oxytocin with the activity of 0.1 IU ("Gideon Richter", Hungary), which was prepared in the Krebs solution with the addition of 0.1 % DMSO. The blocker of nitrogen oxide synthases N^{ω}-nitro-L-arginine methyl ester (L-NAME, "Sigma") was dissolved in the Krebs solution (or the high-potassium solution, depending on the experiment design) with the administration of 0.1 % DMSO and used in the concentration of 10^{-4} M.

The mechanokinetic analysis of myometrium preparation contractions. The spontaneous contractile activity of myometrium preparations was studied using the method of multiparameter mechanokinetic analysis (Kosterin *et al.*, 2021), according to which the complete profile of specific contraction-relaxation cycles was linearized within the coordinates (where *f* and *t* are instant values of force and time at the level of the contraction cycle, and indices C and R mark the phases of contraction and relaxation, respectively). The linearization charts were used to determine the characteristic

constants *k* and *n*, which were further used to calculate the following 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). Here, V_C and V_R are the maximal velocities of the contraction and relaxation phases, respectively, and I_0, I_C, and I_R are force impulse parameters at the level of the amplitude and maximal velocities of the contraction and relaxation phases, respectively.

The kinetic analysis of spontaneous and induced (by high-potassium solution and oxytocin) contractions of the muscle preparations of rat uterus was conducted by the method of Kosterin–Burdyga with the estimation of normalized maximal velocities of the contraction (V_{nc}) and relaxation (V_{nr}) phases (Burdyga & Kosterin, 1991).

Statistical analysis. The experimental data were processed by variation statistics methods using Origin 2018 and Excel programs. 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 significant differences between the mean values of two samplings (in the control and in the presence of calix[4]arene C-956). The parametric one-way ANOVA was used to determine the significant differences between the mean values of three samplings (in the control, in the presence of L-NAME, and under the effect of calix[4]arene C-956 with the previous introduction of L-NAME); the post-hoc comparison was made using the Tukey's criterion. In all cases, the results were considered significant 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-criterion; 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 transporting activity of Ca^{2+}, Mg^{2+}-ATPase of the PM under the action of calix[4]arene C-956. During the first stage of the study, we investigated the impact of calix[4]arene C-956 on the transporting activity of Ca^{2+}, Mg^{2+}-ATPase of the PM using our elaborated simulation model.

We demonstrated that calix[4]arene C-956 in the concentration of 100 μM effectively inhibited the transporting activity of Ca^{2+}, Mg^{2+}-ATPase of PM, since no accumulation of Ca ions was observed inside the vesicles (**Fig. 3**).

It should be noted that under the cumulative introduction of EDTA and ionophore A-23187 there was a drop in the fluorescent signal below the initial level. It may be explained by the fact that before the transportation of calcium ions by the Ca^{2+}-pump of the PM, the PM vesicles contained some amount of free Ca^{2+}, which enhanced the fluorescent signal of fluo-4. After the administration of EDTA, all free Ca^{2+} got bound, which, in turn, led to a decrease in the probe fluorescence below the initial level of the signal.

We have previously demonstrated that calix[4]arene C-956 inhibited the ATP-hydrolase activity of Ca^{2+}-pump of the PM. Yet, the inhibition of the enzymatic hydrolysis of ATP is equivocal evidence to the inhibition of the transporting activity as well. Indeed, both our investigations and the obtained results (**Fig. 3**) confirm that C-956 inhibits not only the ATP-hydrolase activity of Ca^{2+}, Mg^{2+}-ATPase, but also the transporting function of Ca^{2+}-pump of the PM in the uterine myocytes.

Using confocal microscopy, we demonstrated that the application of calix[4]arene C-956 to the immobilized myocytes of the uterus caused an increase in the level of the intracellular concentration of Ca ions (the data are not presented) (Veklich *et al*., 2018).

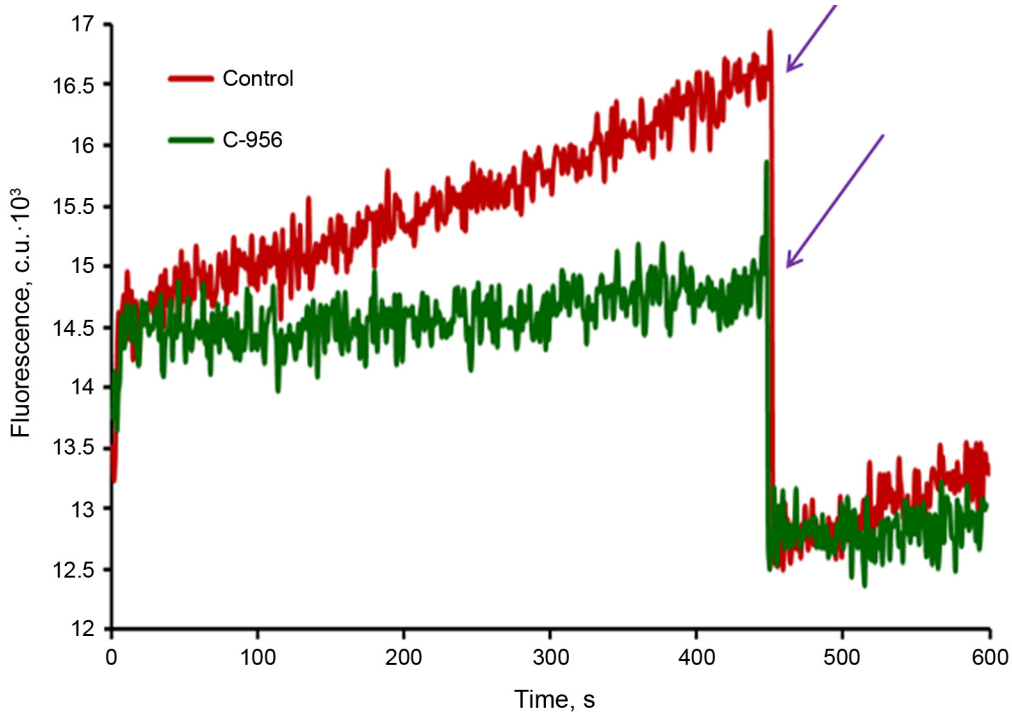

Fig. 3. The impact of calix[4]arene C-956 (100 μM) on Mg^{2+}-ATP-dependent transportation of Ca^{2+} in the PM fraction of the myometrium.
The asterisks indicate the moment of introducing the aliquot of the calcium ionophore A-23187 solution along with EDTA (final concentrations in the incubation medium were 5 μM and 2 mM, respectively). The results of the typical experiment are presented

As seen from the results of the study on the transporting activity of Ca^{2+}, Mg^{2+}-ATPase, calix[4]arene C-956 has a powerful capability of inhibiting this process. However, in light of a prospective use of this compound as a uterotonic, it is no less important to preserve its ability to activate the contractile activity under conditions of the intact tissue as well. Therefore, using the smooth muscle preparations of rat uterine horns under the isometric registration mode, we studied the effects of this compound (at the fixed concentration of 10^{-5} M) on the mechanokinetics of the spontaneous contractile activity and the one, induced via electro- and pharmacomechanical conjugation.

The regularities of the impact of calix[4]arene C-956 on the spontaneous activity of rat myometrium. The introduction of 10 μM calix[4]arene C-956 into the Krebs solution and washing the smooth muscle samples did not cause any changes in the basal tension and their spontaneous contractile activity (amplitude and frequency). It was found that after the preliminary incubation of the myometrium preparations with calix[4]arene C-956 (10 μM, for 20–40 min), the average parameters of spontaneous contractile activity as compared to the control values taken as 100 %, were as follows: the amplitude – 113.7±2.3 % ($n = 5$, $p < 0.05$) and the frequency – 116.7±4.6 % ($n = 5$, $p < 0.05$). Thus, at the concentration of 10 μM, calix[4]arene C-956 poorly manifests the ability to modify spontaneous contractions of the myometrium (**Fig. 4**).

Hereinafter, separate spontaneous contractions were analyzed by Kosterin's multiparametric mechanokinetic analysis with the estimation of the following 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). Here, V_C and V_R are the maximal velocities of the contraction and relaxation phases, respectively, and I_0, I_C, and I_R are force impulse parameters at the level of the amplitude and maximal velocities of the contraction and relaxation phases, respectively.

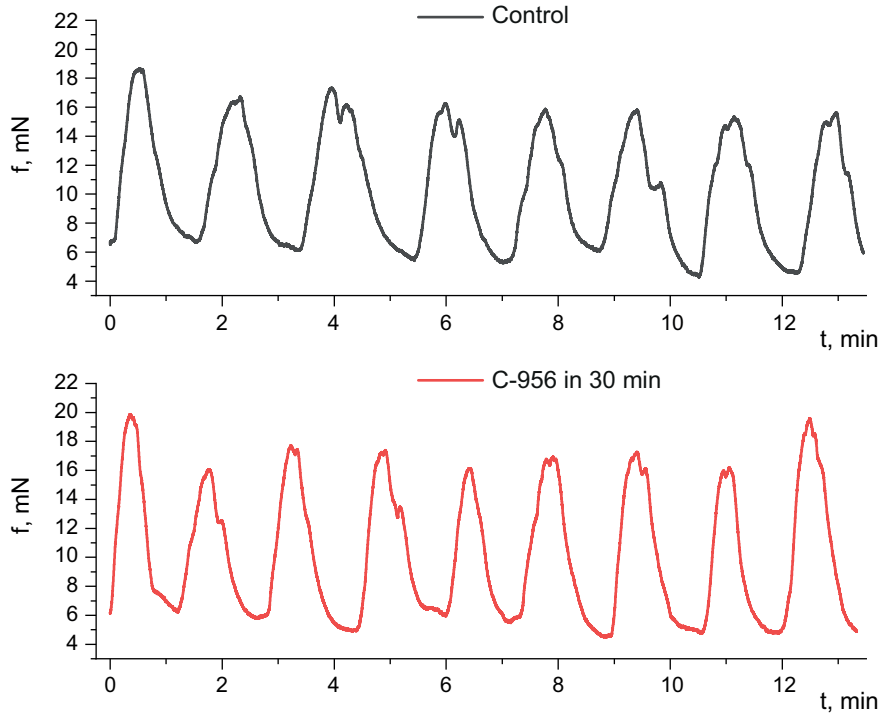

Fig. 4. The spontaneous contractile activity (under the isometric mode of registration) of the rat myometrium in the control and under the effect of calix[4]arene C-956 (10^{-5} M). All experiments were conducted against the background of the DMSO diluent (0.1 %). Typical mechanograms of spontaneous contraction registration are presented

It was found that against the background of C-956, there was a reliable increase in other force parameters of the spontaneous contractions in addition to F_{max} (**Fig. 5*A***). For instance, the parameter of the force for the maximal velocity of the relaxation phase (F_R) was reliably increasing as compared to the control, on average amounting to 113.9±2.0 % (n = 5, p <0.05); and the similar parameter of the contraction phase (F_C) tended to increase (on average up to 111.4±4.9 %, n = 5, p <0.05).

At the same time, under the effect of calix[4]arene C-956, there was a considerable and even (about one quarter compared to the control) decrease in the indices of all the time parameters. For instance, the index of the time of reaching the amplitude (τ_0) and time parameters, at which the maximal velocities of the phases of contraction (τ_C) and relaxation (τ_R) were reached, were as follows: 78.0±3.2 % (n = 5, p <0.05), 74.2±7.7 % (n = 5, p <0.05) and 78.9±2.3 % (n = 5, p <0.01), respectively, as compared to the control parameters (**Fig. 5*B*).**

Since the C-956 compound caused opposite effects in terms of time (decrease) and amplitude (increase) parameters, it had an impact on the formation of quantitative values of the impulse parameters, which did not have any considerable differences regarding the relevant control indices, though manifested some tendencies towards the decrease (**Fig. 5*C*).** For instance, the force impulse on the level of amplitude (I_0) was 89.1±5.3 % (n = 5, p >0.05) on average, and the impulse on the level of the maximal velocity of the phases of contraction (I_C) and relaxation (I_R) were 83.2±12.2 % and 90.0±3.7 % on average, respectively (in both cases n = 5, p >0.05).

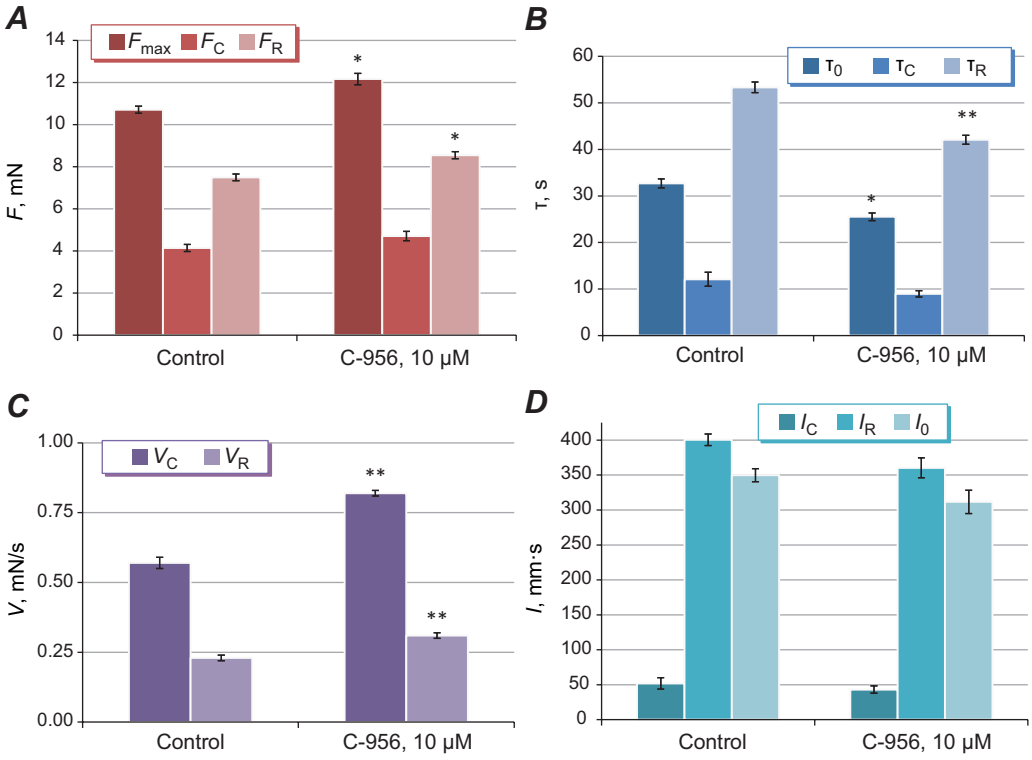

Fig. 5. The summary of mechanokinetic parameters of the spontaneous contractile activity of rat myometrium under the effect of calix[4]arene C-956 (used in the concentration of 10 μM): ***A*** – force parameters (F_{max}, F_C, and F_R); ***B*** – time parameters (τ_0, τ_C, and τ_R); ***C*** – velocity parameters (V_C and V_R); ***D*** – impulse parameters (I_0, I_C, and I_R). n = 5; * – p <0.05, ** – p <0.01 – significant relative to the control

Also, under the effect of calix[4]arene C-956, there was a considerable and even increase in the indices of maximal velocities of the contraction (V_C) and relaxation (V_R) phases, which on average were 143.2±1.8 % (n = 5, p <0.01) and 137.4±2.1 % (n = 5, p <0.01), respectively (**Fig. 5*D***).

The model, used for the mechanokinetic analysis envisaged the estimation of the absolute values of the maximal velocities V_C and V_R, but it was interesting to compare the changes in the velocity parameters regardless of the amplitude. Therefore, we applied the method of estimating the normalized maximal velocities of the contraction and relaxation phases, elaborated by S. Kosterin and T. Burdyga (Burdyga & Kosterin, 1991).

It was found that C-956 compound also led to a considerable increase in the velocity parameters, independent of the amplitude (**Fig. 6**). For instance, the normalized maximal velocity of the contraction phase (V_{nc}) was on average 126.4±3.0 % (n = 5, p <0.05) and the normalized maximal velocity of the relaxation phase (V_{nr}) was 120.2±0.6 % (n = 5, p <0.05) on average as compared to the control values, accepted as 100 %.

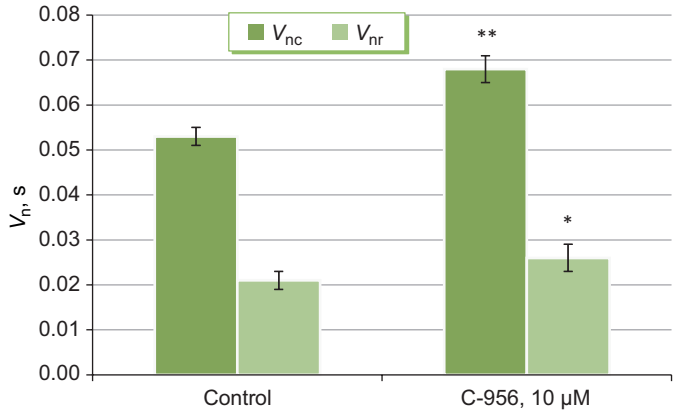

Fig. 6. The normalized maximal velocities of the contraction and relaxation phases (V_{nc} and V_{nr}, respectively) of the spontaneous contractions of rat myometrium under the effect of calix[4]arene C-956 (used in the concentration of 10 μM). n = 5; * – p <0.05, ** – p <0.01 – significant regarding the control

Thus, at the applied concentration of 10 μM, calix[4]arene C-956 poorly manifests the ability to modify spontaneous contractions of the myometrium, including the modulation of the relaxation process.

The mechanokinetic effects of the impact of calix[4]arene C-956 on the spontaneous contractile activity of rat myometrium in the case of blocking nitrogen oxide synthases. It is known that in addition to the ion transporting role, PMCA is also involved in the formation of caveolar signalling protein complexes in such a way that functionally active PMCA molecules block the work of nitrogen oxide synthases, and vice versa, when PMCA is blocked, there is a considerable increase in NO synthesis in cells (Schnitzer *et al*., 1995; Floyd & Wray, 2007; Liu *et al*., 2007). NO molecules induce a tocolytic effect that not only reduces the amplitude of contractions, but also considerably accelerates the process of myometrium relaxation (Srinivasan *et al*., 2021). Thus, we tested the assumption about a possible contribution of NO to inhibiting the amplitude of spontaneous contractions of rat myometrium in the presence of calix[4]arene C-956. The non-selective inhibitor of NO-synthases, L-NAME, was used in the study.

The following experiments were designed to investigate the effects of calix[4]arene C-956 (10 μM) on the background of preliminary NO-synthase blocking by L-NAME compound (0.1 mM, preliminary incubation period: 30 min) (**Fig. 7**).

In the case of the blocked NO synthesis, the effects of calix[4]arene C-956 were more pronounced. For example, the application of L-NAME demonstrated a tendency towards an increase in the amplitude of spontaneous contractions. The introduction of calix[4]arene C-956 induced an increase in the amplitude up to 161.9 ± 5.2 % on average (compared to the control, accepted as 100 %, n = 5; p <0.01) (**Fig. 8**).

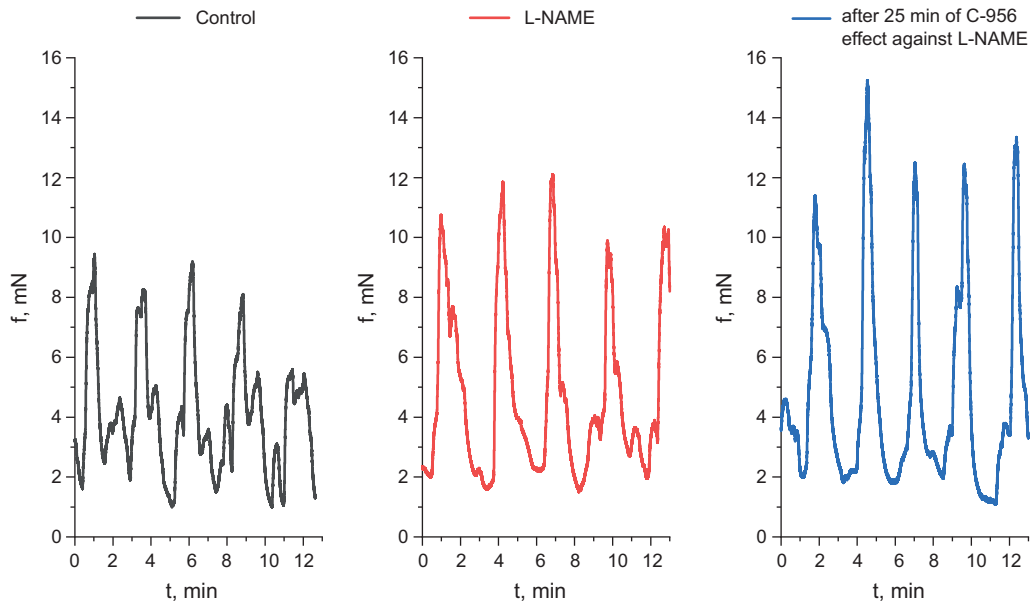

Fig. 7. The spontaneous contractile activity of rat myometrium in the control, on the background of the effect of the non-selective blocker of nitrogen oxide synthases, L-NAME (10^{-4} M, the preliminary incubation lasted 30 min) and on the background of the effect of calix[4]arene C-956 (10^{-5} M, 25 min after the start of the application) under preliminary incubation of the muscle preparations with L-NAME. All experiments were conducted on the background of the DMSO diluent (0.1 %).
Typical mechanograms of spontaneous contraction registration are presented

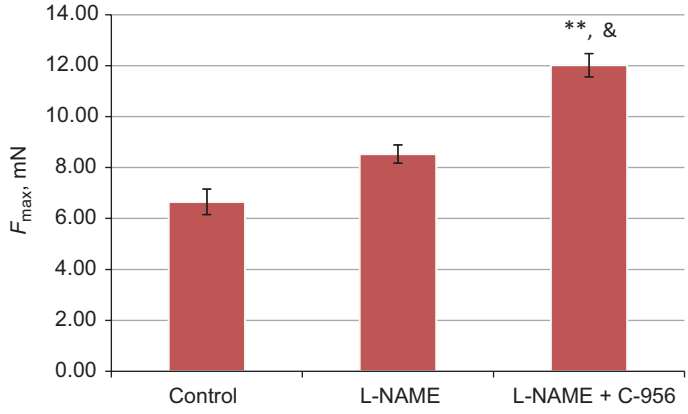

Fig. 8. The amplitude (F_{max}) of spontaneous contractions of rat myometrium in the control, on the background of the effect of the non-selective blocker of nitrogen oxide synthases, L-NAME (10^{-4} M, the preliminary incubation : 30 min) and on the background of the effect of calix[4]arene C-956 (10^{-5} M, 25 min after the start of the application) under preliminary incubation of the muscle preparations with L-NAME. n = 5; ** – p <0.01 – regarding the control and & – p <0.05 – significant relative to L-NAME

Then, method of multiparameter mechanokinetic analysis was used to analyze specific spontaneous contractions, registered under the action of L-NAME (10^{-4} M, the contractions registered 30 min after the start of applying the substance were under analysis). It was determined that the immediate blocking of NO synthesis was accompanied with

a significant increase in the amplitude parameters of spontaneous contractions: F_{max}, F_C, and F_R were 128.3±4.4 %, 132.8±3.6 % and 116.3±5.7 %, respectively (regarding the control indices, accepted as 100 %, n = 5; in all cases p <0.05) (**Fig. 9*A***). It is noteworthy that the subsequent addition of calix[4]arene C-956 (10^{-5} M) to the washing solution led to a further increase in the amplitude parameters, which was considerable in case of F_{max}, F_C, and F_R compared to the effects of L-NAME alone (n = 5; in all cases p <0.01 regarding the control and p <0.05 regarding L-NAME).

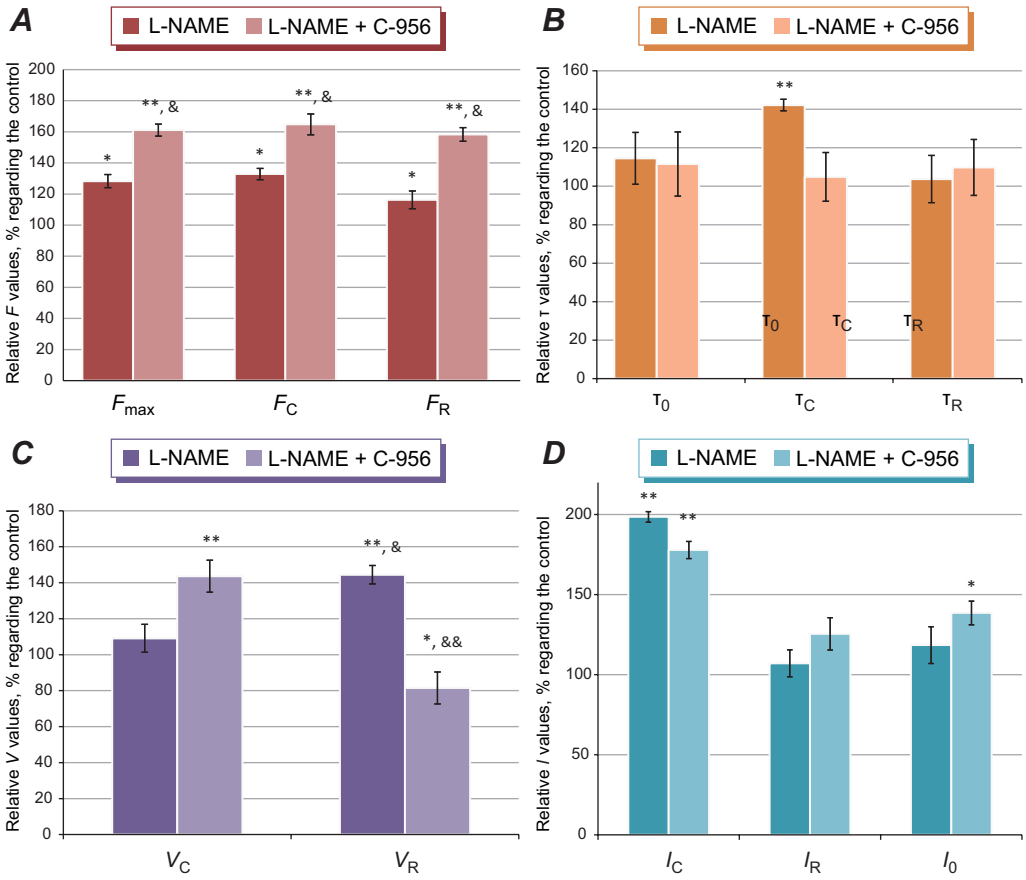

Fig. 9. The summary of relative (regarding the control, accepted as 100 %) mechanokinetic parameters of the spontaneous contractile activity of rat myometrium on the background of the effect of the non-selective blocker of nitrogen oxide synthases, L-NAME (10^{-4} M, the preliminary incubation lasted 30 min) and on the background of the effect of calix[4]arene C-956 (10^{-5} M, 25 min after the start of the application) under preliminary incubation of the muscle preparations with L-NAME: ***A*** – force parameters (F_{max}, F_C, and F_R); ***B*** – time parameters (τ_0, τ_C, and τ_R); ***C*** – velocity parameters (V_C and V_R); ***D*** – impulse parameters (I_0, I_C, and I_R). n = 5; * – p <0.05 and ** – p <0.01 – significant regarding the control, & – p < 0.05 and && – p <0.01 regarding L-NAME

The index of the characteristic time of the contraction phase τ_C increased considerably in the presence of L-NAME (up to 142.2±3.0 % regarding the control, n = 5, p <0.01). However, there were no changes in other time parameters against the background of L-NAME alone or in combination with calix[4]arene C-956 (**Fig. 9*B***).

In addition, L-NAME selectively changed the velocity parameters of spontaneous contractions: the velocity V_C remained at the control level, whereas V_R significantly increased up to 144.4±5.1 % (n = 5, p <0.01). In the context of inhibiting NO synthesis, calix[4]arene C-956 induced notable changes in both velocity parameters: V_C increased (on average up to 143.6±8.9 % relative to the control, n = 5, p <0.01) whereas V_R decreased significantly (on average down to 81.5±7.2 % relative to the control, n = 5, p <0.05) (**Fig. 9*C***).

The estimation of impulse parameters under the action of L-NAME demonstrated that blocking NO synthases led to an increase in I_C only, whereas I_0 and I_R remained at the control level. The application of calix[4]arene C-956 in combination with L-NAME was accompanied with a considerable increase in I_C and I_0 parameters, whereas the impulse on the level of the characteristic time of the relaxation phase I_R was at the control level (**Fig. 9*D***).

Besides, the preliminary incubation of the myometrium preparations with L-NAME (10^{-4} M) had a considerable effect on the parameters of the normalized maximal velocities: in the contraction phase, there was a decrease in V_{nc} down to 79.5±2.8 % compared to the control (n = 5, p <0.05), while the parameter V_{nr} decreased on average down to 45.1±2.4 % (n = 5, p <0.001) compared to the control, accepted as 100 % (**Fig. 10**).

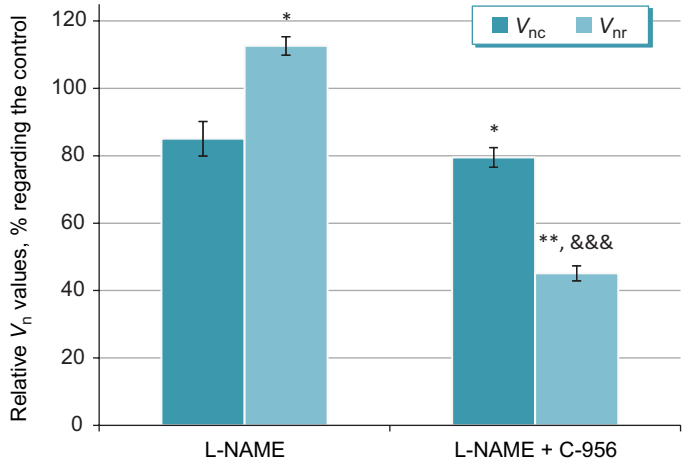

Fig. 10. The relative values of the normalized maximal contractions of the phases of contraction (V_{nc}) and relaxation (V_{nr}) of spontaneous contractions in the rat myometrium in the control, under the blocking of nitrogen oxide synthases by L-NAME (10^{-4} M, the preliminary incubation: 30 min) and under the effect of calix[4]arene C-956 (10^{-5} M, the preliminary incubation with C-956: 25 min) in the context of blocking the nitrogen oxide synthases with L-NAME (10^{-4} M). n = 5; * – p <0.05 and ** – p <0.01 relative to the control, &&& – p <0.001 relative to L-NAME

Additionally, calix[4]arene C-956, which, as shown by the enzymatic study on the preparations of the myocyte PMs, is a highly affine inhibitor of Ca^{2+}-pump of the PM (Veklich *et al*., 2018), at the fixed concentration of 10^{-5} M has a considerable effect on the mechanokinetics of spontaneous contractile activity of the rat myometrium, considerably slowing down the relaxation phase of the spontaneous isometric contractions. It was found that the effects of C-956 on the spontaneous contractions were considerably modulated in the case of blocking nitrogen oxide synthesis. These effects are likely to

be related to the inhibition of the extrusion of Ca^{2+} ions from the cytosol of myocytes via PMCA and enhancing nitrogen oxide synthesis with constitutive NOS (Schuh *et al*., 2001; Srinivasan *et al*., 2021).

The modulation of the contractions in the rat myometrium induced by high-potassium depolarization using calix[4]arene C-956. At the subsequent stage, we investigated the regularities in the effect of calix[4]arene C-956 on the excitations-contractions in the rat myometrium induced via the pathway of electromechanical conjugation, which was modelled using the high-potassium solution (80 mM).

It is known that under this technique of activation, the contractions in myocytes are achieved via the release of Ca^{2+} ions through voltage-gated Ca^{2+}-channels, and the processes of decreasing the intracellular concentration of this cation (at the level of the relaxation phase) occur due to the functioning of the energy-dependent transportation systems (Matthew *et al*., 2004; Kishor Kumar *et al*., 2024).

In case of isometric contractions in the myometrium, activated by the high-potassium solution (80 mM), calix[4]arene C-956 (10^{-5} M) induced an increase in the amplitude up to 123.4±4.6 % compared to the control, accepted as 100 % (n = 5, p <0.05) (**Fig. 11**).

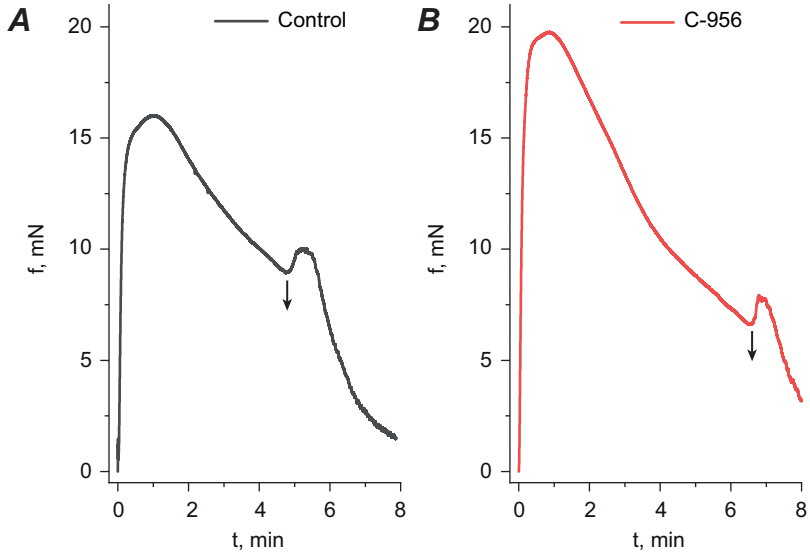

Fig. 11. The comparison of isometric contractions of the myometrium induced by the application of a high-potassium solution (80 mM) in the control (**A**) and under the effect of calix[4]arene C-956 (10^{-5} M) (***B***). All experiments were conducted on the background of the DMSO diluent (0.1 %). The moment of the start of washing muscle preparations from the high-potassium solution is shown with asterisks

The kinetic analysis demonstrated that under the action of C-956, the normalized maximal velocity of the contraction phase V_{nc} remained at the control level, whereas the velocity of relaxation V_{nr} decreased on average down to 85.1±3.7 % (n = 5, p <0.05).

Further experiments were conducted in the context of preliminary NO-synthases blocking by L-NAME compound (0.1 mM, the preliminary incubation: 30 min) (**Fig. 12**). The application of L-NAME increased the maximal force of contraction up to 131.5±4.2 % (n = 5, p <0.05) relative to the control, whereas the parameters of the normalized

maximal velocity of the contraction and relaxation phases V_{nc} and V_{nr} were at the level of the control indices (94.8 % and 93.5 % on average, respectively).

Under the effect of L-NAME, calix[4]arene C-956 induced an increase in the maximal force of contraction, which on average was 111.8±3.6 % (n = 5, p >0.05) relative to L-NAME and 146.9±4.9 % (n = 5, p <0.05) relative to the control.

The mechanokinetic analysis demonstrated that the normalized maximal velocity of the contraction phase V_{nc} was at the control level (on average 109.1±4.4 %; n = 5, p >0.05). The normalized maximal velocity of the relaxation phase V_{nr} under the effect of C-956 decreased considerably and, on average, was 58.1±4.2 % (n = 5, p <0.01) relative to the effect of L-NAME and 54.3±3.5 % (n = 5, p <0.001) relative to the control.

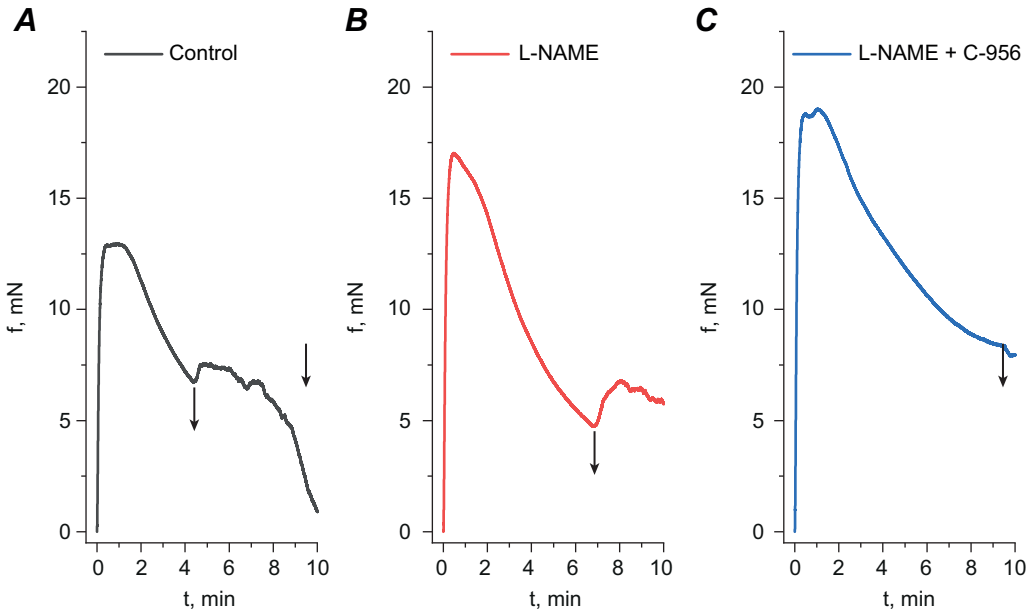

Fig. 12. The comparison of the myometrium contractions, induced by the application of a high-potassium solution (80 mM), on the background of NO synthase blocking in the control (Control, ***A***), on the background of NO synthase blocking using the preliminary incubation with L-NAME (L-NAME, ***B***) (0.1 mM, 30 min) and under the action of calix[4]arene C-956 (10^{-5} M) on the background of NO-synthase blocking (L-NAME + C-956, ***C***). All experiments were conducted on the background of the DMSO diluent (0.1 %). The moment of the start of washing muscle preparations from the high-potassium solution is shown with asterisks

The modulation of the contractions in the rat myometrium, induced by oxytocin, using calix[4]arene C-956. The aim of the subsequent stage was to determine the regularities in the effect of calix[4]arene C-956 on the excitations-contractions in the rat myometrium, induced via the pathway of pharmacomechanical conjugation. The pharmacomechanical conjugation was modelled using the uterotonic hormone, oxytocin (0.1 IU).

In case of isometric contractions in the myometrium, activated by oxytocin (0.1 IU) on the background of the preliminary incubation with calix[4]arene C-956 (10^{-5} M, the duration of the preliminary incubation: 30 min), there was a considerable increase in their amplitude up to 125.3 ± 4.8 % on average compared to the control, accepted as 100 % (n = 5, p <0.05) (**Fig. 13**).

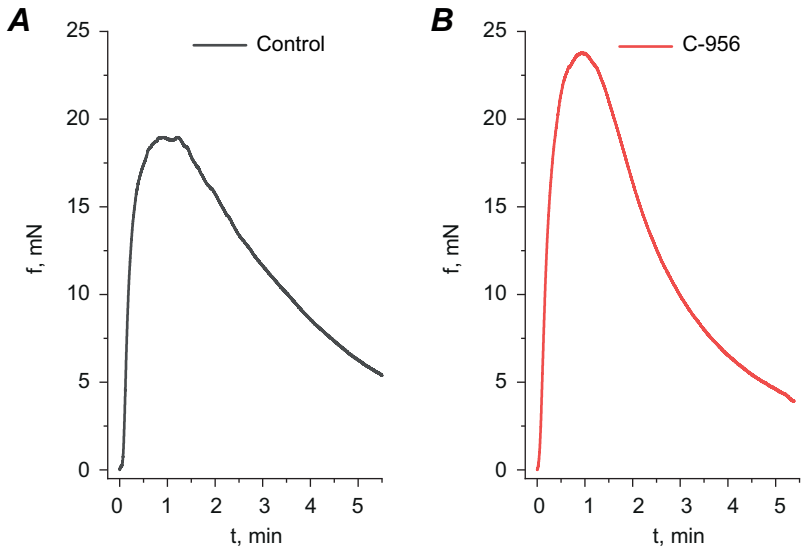

Fig. 13. The comparison of myometrium contractions, induced by the application of oxytocin (0.1 IU) in the control (***A***) and under the effect of calix[4]arene C-956 (10^{-5} M) (***B***). All experiments were conducted on the background of the DMSO diluent (0.1 %). Typical mechanograms are presented

The kinetic analysis demonstrated that under these conditions, the normalized maximal velocity of the contraction phase V_{nc} remained at the control level, whereas against the background of C-956, the normalized maximal velocity of the relaxation phase V_{nr} increased on average up to 125.9±2.1 % (n = 5, p <0.05).

The effects of calix[4]arene C-956 against the background of L-NAME compared to the oxytocin-induced contraction did not have any considerable differences regarding the effects registered under the action of L-NAME alone (**Fig. 14**): the maximal force of contraction was 104.8±5.1 % (relative to the effect of L-NAME, n = 5, p >0.05), and the mechanokinetic parameters were partially changed. For instance, on average, the velocity of the contraction phase (V_{nc}) was 121.3±4.4 % relative to the effect of L-NAME (n = 5, p <0.05) and 106.6 ± 3.7 % relative to the control (n = 5, p >0.05), and the normalized maximal velocity of relaxation (V_{nr}) was on average 116.0±4.8 % relative to the effect of L-NAME and 105.1±5.3 % relative to the control (in both cases n = 5, p >0.05).

It is also noteworthy that under the effect of calix[4]arene C-956 on the background of L-NAME, the level of the residual contraction (the so-called tonic component of oxytocin-induced contraction) was notably increased. It may indicate the increased level of Ca^{2+} ions in the cytoplasm, likely induced by calix[4]arene C-956 as a result of blocking PMCA and, possibly, partially the Ca^{2+}-pump of the sarcoplasmic reticulum.

The C-956 compound triggers the activation of oxytocin-induced contractions, which are characterized by the increase in the normalized maximal velocity of the relaxation phase (V_{nr}) . The preliminary blocking of NO-synthases eliminates the effect of calix[4]arene C-956 regarding V_{nr} and is accompanied with an increase in the tonic component of the oxytocin-induced contraction, which possibly confirms the ability of this compound to block the Ca^{2+}-pump of PM and induce the maintenance of the increased concentration of Ca^{2+} ions in the cytoplasm of smooth muscle cells of the myometrium (Veklich & Kosterin, 2005).

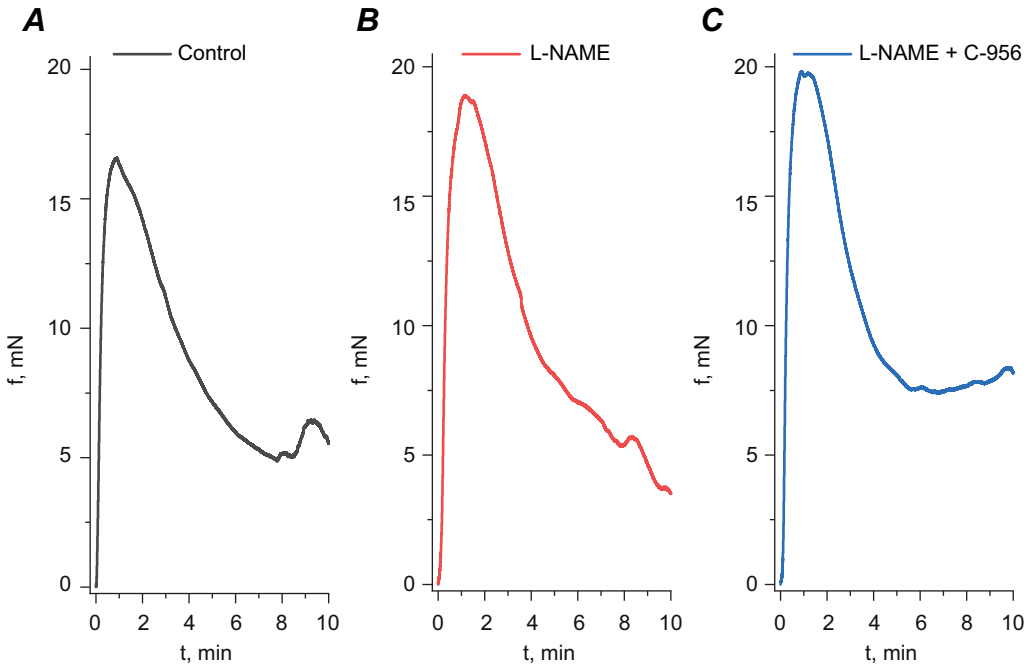

Fig. 14. The comparison of the myometrium contractions induced by the application of oxytocin (0.1 IU): in the control (***A***), on the background of NO synthases blocking using the preliminary incubation with L-NAME (0.1 mM, 30 min) (***B***) and under the effect of calix[4]arene C-956 (10^{-5} M) on the background of NO-synthase blocking by L-NAME (0.1 mM) (***C***). All experiments were conducted on the background of the DMSO diluent (0.1 %). Typical mechanograms are presented

CONCLUSIONS

Our study has demonstrated that in addition to the ability to inhibit ATP-hydrolase activity of Ca^{2+}, Mg^{2+}-ATPase, calix[4]arene C-956 also induces the blocking of the transporting function of PMCA in uterine myocytes.

Under a short-term effect on myometrium preparations, the compound C-956 induces the modulation of spontaneous contractile activity, including an increase in the frequency and amplitude against the background of the change in some mechano-kinetic parameters of specific spontaneous contractions. NOS blocking considerably enhanced the effects of calix[4]arene C-956 on the amplitude of spontaneous contractions, which is in good agreement with the scientific literature data about the structural-functional connections between the molecules of PMCA and NOS. It can be reasonably assumed that the blocking of the Ca^{2+}-pump increases the production of nitric oxide, which is a muscle relaxant. Therefore, the increase in the concentration of $[Ca^{2+}]_i$ under the influence of C-956 was not significantly reflected in the contractile activity of the myometrium. Conversely, with the previous inhibition of NOS followed by the use of C-956, we were able to fully demonstrate the contribution of PMCA to the regulation of the contractile activity of the uterus. Additionally, calix[4]arene C-956 demonstrated a considerable impact on the contractions induced by the high-potassium depolarization of the plasma membrane and by oxytocin, increasing their amplitude and decrea-

sing the relaxation velocity. This evidence proves that this compound can modulate the processes of electro- and pharmacomechanical coupling of excitation-contraction in smooth uterine muscles which is crucial for the understanding of its potential utility as a uterotonic.

The findings of this study demonstrate the potential of calix[4]arene C-956 as a regulator of contractile activity in the myometrium, which could be used in clinical practice to regulate the labor activity and treat the dystonia of smooth uterine muscles.

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

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

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КАЛІКС[4]АРЕН С-956 ЯК СЕЛЕКТИВНИЙ ІНГІБІТОР Ca^{2+}-ПОМПИ ПЛАЗМАТИЧНОЇ МЕМБРАНИ ТА МОДУЛЯТОР СКОРОЧУВАЛЬНОЇ ФУНКЦІЇ МІОМЕТРІЯ

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Обґрунтування. На сьогодні є важливим створення і тестування фармакологічних інструментів для селективного інгібування кальцієвої помпи плазматичної мембрани як основи лікарських препаратів, зокрема, для терапії порушення збудливості серцевого та гладеньких м'язів. У попередніх дослідах ми з'ясували, що калікс[4]арен С-956 ефективно пригнічує Ca^{2+},Mg^{2+}-АТФ-азну активність препаратів плазматичних мембран клітин міометрія. Мета цієї роботи – дослідити закономірності й механізми впливу калік[4]сарену С-956 на Ca^{2+}-транспортувальну активність Ca^{2+},Mg^{2+}-АТФ-ази плазматичної мембрани та скорочувальну функцію міометрія щурів.

Матеріали та методи. Експерименти проводили на безпородних білих щурах. Досліджували Ca^{2+}-транспортувальну активність препаратів суспензії плазматичних мембран міоцитів, навантажених Ca^{2+}-чутливим флуоресцентним зондом fluo-4 AM. Реєстрацію скорочувальної активності на препаратах поздовжніх гладеньких м'язів рогів матки зі збереженим ендотелієм проводили в ізометричному режимі.

Результати. Встановлено, що калікс[4]арен С-956 спричиняє блокування транспортної функції кальцієвої помпи препаратів плазматичних мембран міоцитів матки. Сполука С-956 за короткотривалої дії на багатоклітинні препарати міометрія щурів спричиняє підвищення амплітуди спонтанних скорочень і зміну їхніх механокінетичних параметрів. Також калікс[4]арен С-956 суттєво впливає на скорочення, спричинені гіперкалієвою деполяризацією плазматичної мембрани

й окситоцином, підвищуючи їхню амплітуду і знижуючи швидкість розслаблення. Блокування синтезу оксиду азоту суттєво посилює ефекти С-956 на спонтанні та зумовлені гіперкалієвим розчином і окситоцином скорочення препаратів міометрія.

Висновки. Отримані нами результати дають змогу передбачити, що головною мішенню впливу калікс[4]арену С-956 на міоцити є кальцієва помпа плазматичної мембрани. За попереднього інгібування синтаз оксиду азоту з подальшим використанням С-956 ми отримали змогу вповні виявити внесок кальцієвої помпи плазматичної мембрани в регуляцію скорочень матки.

Ключові слова: міометрій, Ca^{2+},Mg^{2+}-АТФ-аза плазматичної мембрани, калікс[4]арен С-956, скорочення, механокінетичний аналіз, Ca^{2+}-сигнал

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