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THAPSIGARGIN-RESISTANT THIACALIX[4]ARENE C-1087-SENSITIVE COMPONENT OF THE CONTRACTILE ACTIVITY IN RAT MYOMETRIUM REFLECTS THE FUNCTIONING OF PLASMA MEMBRANE CALCIUM PUMP

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Background. According to existing knowledge, thiacalix[4]arene C-1087 is highly capable of inhibiting Ca^{2+}-pump (Ca^{2+}, Mg^{2+}-ATPase) of the plasma membrane; at the same time it inhibits the functioning of Ca^{2+}-pump of the sarcoplasmic reticulum of uterine smooth muscles to some degree. The aim of this research was to study the effects of C-1087 on the concentration of Ca^{2+} ions and contractile activity of the rat myometrium cells using an inhibitor of Ca^{2+}-pump of the sarcoplasmic reticulum – thapsigargin.

Materials and Methods. The experiments were conducted using outbred white non-pregnant rats. The contractile activity in the preparations of longitudinal SM of uterine horns with preserved endothelium was registered in the isometric mode. To determine the changes in $[Ca^{2+}]_i$ level, myocytes were treated with probes Hoechst 33342 (to test the nucleus of the cell) and fluo-4 AM (to test the change in Ca^{2+}-concentration in the cell).

Results. The tenzometric studies with the subsequent mechanokinetic analysis demonstrated that under the action of thapsigargin (0.5 μM), thiacalix[4]arene C-1087 (10 μM) caused considerable changes in the kinetics of the spontaneous contractile activity processes in the myometrium of rats, including the decrease in the maximal contraction velocity and the increase in the maximal relaxation velocity. By means of confocal microscopy with Ca^{2+}-sensitive fluorescent probe fluo-4, it was demonstrated that the application of thiacalix[4]arene C-1087 to immobilized myocytes of the uterus

against the background of thapsigargin caused a transient spike of Ca^{2+}-signal with the subsequent turn of the intracellular concentration of Ca ions to the stable increased level. The effects of C-1087 under the action of thapsigargin regarding the relaxation phase in the spontaneous myometrium contractions were removed after the preliminary blocking of nitric oxide synthases L-NAME (100 μM). Under the action of L-NAME, thiacalix[4]arene C-1087 (10 μM) caused complete inhibition of the relaxation process in the contraction of myometrium preparations, induced by high-potassium solution (80 mM).

Conclusions. The primary reason for changes in the contractile activity and Ca^{2+}-signal in uterine myocytes under the effect of thiacalix[4]arene C-1087 is its ability to inhibit Ca^{2+}-pump of the plasma membrane; further C-1087-induced changes in the smooth muscle tissues may be caused by the increased level of Ca^{2+} concentration in myocytes. The obtained results demonstrate thiacalix[4]arene C-1087 is a promising compound for the elaboration of pharmacological preparations for modulating the contractile activity in smooth muscles, including myometrium.

Keywords: Ca^{2+},Mg^{2+}-ATPase of the plasma membrane, thiacalix[4]arene C-1087, spontaneous contractions, Ca^{2+}-signal

INTRODUCTION

Myometrium belongs to phasic smooth muscles (SM) and is capable of generating spontaneous potentials of action which induce the contraction process. Contrary to SM of the gastrointestinal tract, where the source of spontaneous activity is the interstitial pace-maker cells of Cajal (ICC), the spontaneous depolarization of the myometrium tissue occurs in the very smooth muscle cells (SMC), but the frequency of spontaneous depolarization waves is modulated by their own ICC-like cells (Hutchings *et al.*, 2009; Bru-Mercier *et al.*, 2012). The contractile activity of this tissue is regulated with hormones (mostly oxytocin), as well as neuromediators and paracrine mediators (including ATP, acetylcholine, prostaglandin $F_{2\alpha}$) (Darios *et al.*, 2012; Tommaso *et al.*, 2017).

The level of myometrium excitability is determined by ion transport (Ca^{2+}, Na^{+}, K^{+}, Cl^{-}) via the plasma membrane (PM) of SMC and the membranes of other organelles – sarcoplasmic reticulum (SR) and mitochondria (MCh). The main source of Ca^{2+} ions in the spontaneous processes of excitation is the penetration of these cations via L-type potential-governed Ca^{2+}-channels of PM (Matthew *et al.*, 2004; Noble *et al.*, 2009; Wray & Arrowsmith, 2021). The release of Ca^{2+} via inositol-1,4,5-triphosphate-governed channels from SR plays a modulatory role and is critical in case of activating the contractions by agonists (Noble *et al.*, 2009; Noble *et al.*, 2014).

The key significance in maintaining the myometrium excitability is attributed to the systems of active Ca^{2+} ion transportation in SMC – Ca^{2+}-pumps of PM and sarco(endo) plasmic reticulum (PMCA and SERCA, respectively) (Floyd & Wray, 2007; Kosterin *et al.*, 2016; O'Day & Huber, 2022; Krebs, 2023). PMCA plays the role of the main system, aimed at maintaining the basal concentration of Ca^{2+}; it facilitates the pumping-out of about 70 % of these cations from the cytoplasm of SMC after excitation (Darios *et al.*, 2012; Wray & Arrowsmith, 2021). Isoforms PMCA 1b, 4a, and 4b are expressed in the myometrium of rats; C-terminal fragment of PMCA molecule, form b of all PMCA isoforms, corresponds to the consensus aminoacid sequence of PDZ-domain. PDZ-domain ensures the interaction between PMCA and intracellular proteins (including guanylate cyclase

MAGUK, constitutive isoforms of nitric oxide synthases nNOS and eNOS) (Hutchings *et al.*, 2009; Corradi *et al.*, 2021). It should be noted that PMCA is concentrated in caveolas (for instance, at a minimal distance from SR), and the stechiometry of this enzyme location in extra- and intracaveolar parts of PM is 1:25 (Floyd & Wray, 2007; Krebs, 2022). At present, there is no doubt that caveolar molecules of PMCA take part in the formation of specific signalosome complexes, and Ca^{2+}-dependent enzymes located here (including NOS) are in the inhibited state during the functioning of pump (Hutchings *et al.*, 2009; Kosterin *et al.*, 2016; Heo *et al.*, 2023).

Three SERCA isoforms are expressed in the uterine tissue: 2a, 2b, and 3; the first two dominate in SMC, while SERCA3 is mainly endothelial (Floyd & Wray, 2007; Wan Omar et al., 2019). Blocking of SERCA using its selective inhibitor – cyclopiazonic acid – induces the increase in the intracellular concentration of Ca^{2+} in SMC of the myometrium along with the changes of phase contraction parameters: the increase in their amplitude and duration (Floyd & Wray, 2007).

Although PMCA and SERCA molecules have structural and functional similarities, they are inhibited by different compounds. For instance, selective inhibitors of SERCA, thapsigargin and cyclopiazonic acid, are convenient instruments in determining its role (and the role of SR) in Ca^{2+}-transporting and Ca^{2+}-regulated processes (Michelangeli & East, 2011; Jaskulska *et al.*, 2020; Christensen *et al.*, 2021). At present, if reliable selective inactivation of PMCA is required, the knockout of genes encoding this pump is applied; and the promising substances for the pharmacological inhibition of PMCA are peptides, caloxines, and macrocyclic compounds, calix[4]arenes (Szewczyk *et al.*, 2008; Tsymbalyuk & Kosterin, 2013; Veklich *et al.*, 2014; Veklich, 2016; Kosterin *et al.*, 2019; Tsymbalyuk, 2021; Boutin *et al.*, 2022). As for calix[4]arene C-90 and its structural analogs, our previous studies provided evidence of their ability to inhibit Ca^{2+}, Mg^{2+}-ATPase activity of myometrium PM preparations much less affecting the enzymatic activity of SERCA (for instance, the values of inhibition coefficient $I_{0.5}$ of calix[4]arene C-90 regarding PMCA and SERCA are 20.2±0.5 μM and 57.0±1.4 μM, respectively) (Veklich *et al.*, 2014; Veklich, 2016; Kosterin *et al.*, 2019). The ability to modulate the contractile activity of uterine SM in rats was also determined for calix[4]arene C-90 (Tsymbalyuk & Kosterin, 2013; Tsymbalyuk, 2021).

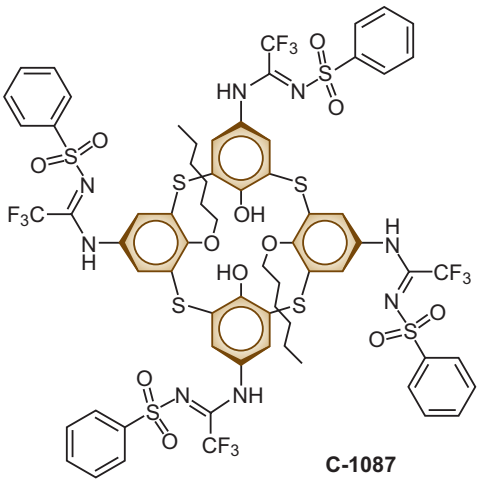
However, the most affine and selective calix[4]arene regarding Ca^{2+}, Mg^{2+}-ATPase of PM is thiacalix[4]arene C-1087. The studies conducted using the suspension models of myometrium membranes and single SMC demonstrate that the values of $I_{0.5}$ regarding the inhibitory effect of thiacalix[4]arene C-1087 on PMCA and SERCA are 20.2±0.5 and >100 μM, respectively (Veklich *et al.*, 2023).

The aim of this study was to investigate thapsigargin-resistant thiacalix[4]arene C-1087-sensitive component of the contractile activity in the myometrium as well as the dynamics of the intracellular concentration of Ca^{2+} ions in uterine myocytes of rats under the condition of “switching off” the calcium pump of SERCA.

MATERIALS AND METHODS

The experiments were conducted using outbred female white rats (the average weight of animals was 200–250 g). All manipulations with animals were carried out in accordance with the International Convention on Animal Welfare and the Law of Ukraine „On Protection of Animals from Cruelty” (Minutes of the meeting of the Commission on Bioethics of NSC „Institute of Biology and Medicine” No 3 of May 2, 2019). Killing of animals was carried out by dislocation of cervical vertebrae under etheric anesthesia.

The structure of thiacalix[4]arene C-1087. Thiacalix[4]arene C-1087 (5,11,17,23-tetra(trifluoro)methyl(phenylsulfonylimino)methylamino-25,27-dihexyloxy-26,28-dihydroxythiacalix[4]arene) (**Fig. 1**) was synthesized and characterized using NMR and infrared spectroscopy methods at the Phosphoranes Chemistry Department (headed by Prof. V. I. Kalchenko, the full member of the NAS of Ukraine) of the Institute of Organic Chemistry, the NAS of Ukraine.



C-1087

Fig. 1. The structural formula of thiacalix[4]arene C-1087

The tenzometric studies of myometrium contractile activity. These studies were conducted by Prof. O. V. Tsymbalyuk at the Institute of High Technologies of the Taras Shevchenko National University of Kyiv. The registration of the contractile activity in the preparations of longitudinal SM of uterine horns with preserved endothelium was done in the isometric mode. Muscle stripes (the average size – 2×10 mm) were placed into the chamber (the volume of 2 mL) with the flowing Krebs solution (the flow rate of 5 mL/min), and thermostated at 37 °C. The preparations were provided with passive tension at the rate of 10 mN and left for one hour until achieving a stable rate of contractions. 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 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 with the equimolar amount of K^+ ions.

Thiacalix[4]arene C-1087 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).

Thapsigargin (the stock concentration in DMSO was 1 mM) in the concentration of 0.5 μM was added to the Krebs solution. The time of preliminary incubation (prior to starting the registration of spontaneous contractions, which were analyzed using the method of mechanokinetic analysis) of the preparations in the flowing Krebs solution with thapsigargin was 20 min.

A non-selective blocker of NO-synthases, L-NAME, was added to the Krebs solution in the concentration of 0.1 mM; the time of preliminary incubation (prior to starting the registration of spontaneous contractions, which were analyzed using the method of

mechanokinetic analysis, see below) of the preparations in the flowing Krebs solution with L-NAME was 20 min.

The control contractile activity and the contractions under the action of L-NAME were studied in the solutions containing 0.1 % DMSO.

The kinetic analysis of the contractile activity. The study of the spontaneous contractile activity in SM preparations was conducted according to the empirical multiparameter method of complex mechanokinetic analysis, previously developed by us (Kosterin *et al*., 2021). To analyze the complete profile of single spontaneous contractions, they were linearized in the coordinates, where *f* and *t* are the instant values of force and time at the level of the contraction cycle (C and R – symbols for the phases of contraction and relaxation, respectively), F_C and F_R – the values of force at the inflexion points of the mechanogram at the contraction phase level (from the beginning of the increase in the force to its maximal value F_{max}) and relaxation (from the maximal value of the force F_{max} at the time moment τ_0 and until its return to the base level), Δt – arbitrarily fixed time interval (which varied within 15–50 s). 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 maximal velocities of the phases of contraction and relaxation, respectively, I_0, I_C and I_R – force impulses at the level of amplitude and maximal velocities of contraction and relaxation, respectively.

Registration of the dynamics of intracellular concentration of Ca^{2+} ions in uterine myocytes. These studies were conducted by professor T. O. Veklich, DSc, and S. O. Karakhim, PhD, using the laser scanning confocal microscope at the Muscle Biochemistry Department (headed by Prof. S. O. Kosterin) of the O. V. Palladin Institute of Biochemistry, the NAS of Ukraine. The myocytes were isolated from the uterus of non-pregnant rats using collagenase and soy inhibitor trypsin by Mollard's method (Mollard *et al*., 1986). The cells were kept in the solution B of the following composition (mM): NaCl – 136.9; KCl – 5.36; KH_2PO_4 – 0.44; $NaHCO_3$ – 0.26; Na_2HPO_4 – 0.26; $CaCl_2$ – 0.03; glucose – 5.5; HEPES (pH = 7.4, 37 °C) – 10.

The SMC suspension for confocal-microscopic studies (the suspension volume of 100 µL) was immobilized for 2 h on the specimen slide and treated with poly-L-lysine (200 µL) at 25 °C. The myocytes, not attached to the slide, were washed off using solution B, then the immobilized cells were added 100 µL of solution B.

To determine the changes in $[Ca^{2+}]_i$, myocytes were treated with probes Hoechst 33342 (to test the nucleus of the cell) and fluo-4 AM (to test the change in Ca^{2+} concentration in the cell). In order to improve the load, the solution also contained 0.2 % pluronic. After 20 min of waiting, the measurements were conducted in the Multi Track mode using the laser scanning confocal microscope LSM 510 META (Carl Zeiss, Germany). For analysis, we selected spindle-shaped cells with a clearly defined nucleus, stained with DNA-sensitive fluorescent probe Hoechst. Thapsigargin (0.1 µM) was added to the medium of myocytes incubation 15 min prior to the registration. The measurements were conducted by consecutive photographing of the cells (time series) for 5 min, 15–20 s each, during which Ca^{2+} (2 mM) was introduced to the medium for myocytes incubation to check the PM integrity. Then the aliquot of the solution of thiacalix[4]arene C-1087 (20 µM) was added. The fluorescence was excited using the diode laser, 405 nm, for Hoechst 33342, registered using the light filter BP, 420–480 nm; the fluorescence of fluo-4 AM was excited by the argon laser, 488 nm, and registered in the range of 505–530 nm (light filter BP 505–530).

Statistical analysis. The statistical analysis of the obtained data was conducted by the methods of variation statistics. The kinetic and statistical calculations were done using the programs MS Excel and Origin 2018.

The samples were checked in terms of belonging to normally distributed general populations according to Shapiro–Wilk's test. The Student's *t*-test was used to determine the significan differences between the two mean values of samplings. The one-way ANOVA was used to determine significan differences between the multiple mean values of samplings; the post-test comparison was made using Scheffe test. In all cases, the results were considered significan on condition of $p < 0.05$. The validation analysis of data approximation by the linear function was performed using Fisher's F-test; determination coefficients (R^2) were at least 0.96 in all cases. The experiment results were presented as the mean ± standard error of mean (M ± SEM), *n* – number of experiments.

RESULTS AND DISCUSSION

Mechanokinetic effects under the conditions of SERCA activity blocking. To determine the role of SERCA in the mechanokinetics of contraction-relaxation processes, we studied the effect of the blocker of this pump, thapsigargin, on the spontaneous contractile activity of rat myometrium.

The introduction of thapsigargin (0.5 μM) into the solution to wash SM preparations was accompanied by the transient increase in the basal tension as well as higher amplitude and frequency of spontaneous contractions (**Fig. 2**). After 20 min of thapsigargin action, the amplitude of contractions remained at the increased level (it was 137.8±4.2 % regarding the control, $p < 0.01$, $n = 7$), but their frequency returned to the control level.

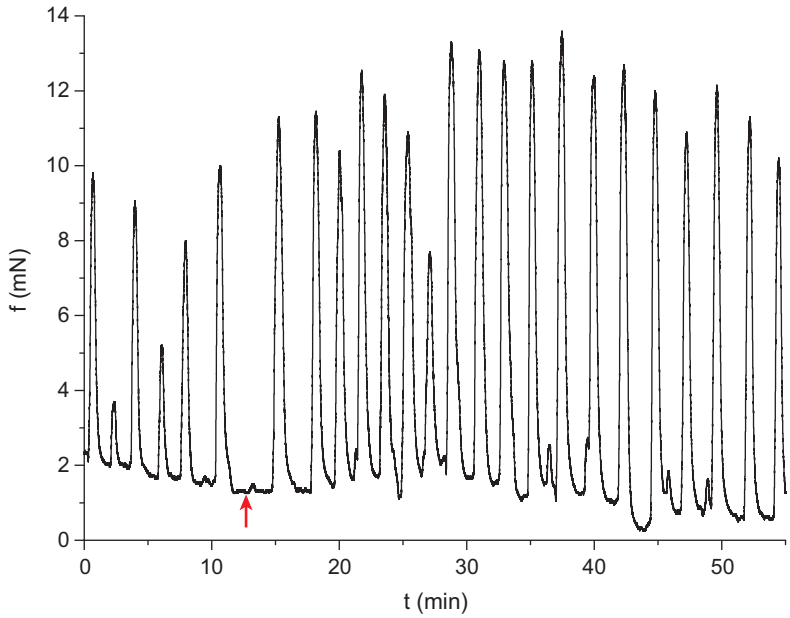


Fig. 2. The change in spontaneous contractile activity in the myometrium of rats under blocking of the sarcoplasmic reticulum calcium pump with thapsigargin (0.5 μM). The moment of thapsigargin introduction into the Krebs solution, smooth muscles washing, is indicated with an arrow. A typical mechanogram is presented

The conducted mechanokinetic analysis demonstrated that thapsigargin changed single cycles of contraction-relaxation considerably, significan increasing the values of force, velocity, and impulse parameters (**Fig. 3**). It should be noted that SERCA blocking did not slow down the process of muscle preparation relaxation, as it could be expected, but led to a significan increase in the maximal velocity of the relaxation phase (V_R).

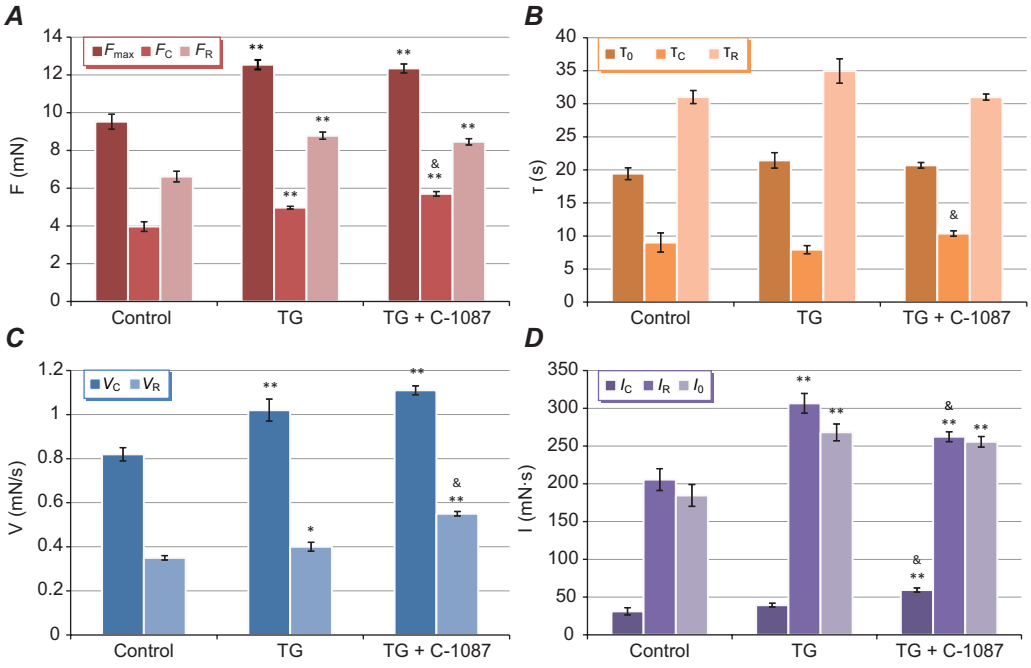


Fig. 3. The mechanokinetic parameters of spontaneous contractions in the rat myometrium in control, under SERCA activity blocking with thapsigargin (TG, 0.5 μM) and the effect of thiacalix[4]arene C-1087 (10 μM) combined with the action of thapsigargin. The parameters are: ***A*** – force (F_{max}, F_C and F_R); ***B*** – time (τ_0, τ_C and τ_R – respectively marked as tau 0, tau C, tau R); ***C*** – velocity (V_C and V_R); ***D*** – impulse (I_0, I_C and I_R). Data were presented as M ± SEM, n = 7. * p <0.05; ** – p <0.01 – compared with control; & – p <0.05 – compared with thapsigargin

It is possible to forecast probable mechanisms by which SERCA blocking leads to the activation of spontaneous myometrium contractions with a complete change in the kinetics of some contraction-relaxation cycles. SR is an intracellular Ca^{2+}-depot which plays the role of the primary source of increasing the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) in the myometrium cells while activating the contractions by agonists-uterotonic drugs and trapping these cations after the effect of the agonists (Taggart & Wray, 1998; Shmigol *et al*., 1999). However, its participation in maintaining spontaneous contractions in uterine SM may be insignificant, mainly limited to the modulation of SMC excitability and partial trapping of calcium ions of SERCA during the relaxation phase with their further vectorial release near PM and thus the activation of Ca^{2+}-dependent K^+-channels (Noble *et al*., 2009; Gravina *et al*., 2010). Probably, the presence of thapsigargin should cause an increase in Ca^{2+}-concentration in the cytosol. According to the data by F. S. Gravina *et al*. (2010), the blocking of SERCA with cyclopiazonic acid causes an increase in $[Ca^{2+}]_i$, related to the enhanced release of these cations from the

extracellular space via potential-governed Ca^{2+}-channels of L-type. Since the blocking of SERCA leads to an increase in $[Ca^{2+}]_i$, including that in the near-membrane space, under these conditions, it is possible to predict the activation of Ca^{2+}-activated membrane proteins (including Ca^{2+}-activated K^+-channels and NO-synthases) (Noble *et al*., 2009; Gravina *et al*., 2010; Pehlivanoğlu *et al*., 2013; Heo *et al*., 2023). This hypothesis has a physiological basis, because the constitutive, Ca^{2+}-activated isoforms of NO-synthase are expressed in the myometrium of non-pregnant rats (mostly the endothelial type and slightly less the neuronal type), while the inducible Ca^{2+}-independent isoform in the non-pregnant state is contained in minimal amounts and dramatically increases during pregnancy (Chatterjee *et al*., 1996; Dong *et al*., 1996). Besides, Ca^{2+}-activated K^+-channels (of large and small conductances) are expressed in the myometrium of rats (Brown *et al*., 2007; Rahbek *et al*., 2014).

Mechanokinetic effects of thiacalix[4]arene C-1087 in combination with SERCA activity blocking. Many studies demonstrate (Bru-Mercier *et al*., 2012; Noble *et al*., 2014; Tommaso *et al*., 2017; Krebs, 2023) that the main system of active pumping-out of Ca^{2+} from the cytoplasm of myometrium cells after the excitation is PMCA. Our previous studies showed that PMCA blocker – thiacalix[4]arene C-1087 in the concentration of 10 μM caused a decrease in the amplitude of spontaneous contractions of rat myometrium (down to 78.4±5.2 % regarding the control, $p < 0.05$, $n = 7$) under the condition of stable frequency.

Thus, in the following series of experiments, we investigated the effect of this compound on mechanokinetic parameters of spontaneous myometrium contractions under the conditions of the previous SERCA blocking (**Fig. 3** and **4**).

Under the conditions of SERCA blocking with thapsigargin (0.5 μM, the time of the preliminary incubation – 20 min), thiacalix[4]arene C-1087 did not impact the amplitude or the frequency of spontaneous contractions (**Fig. 4*A***), but it caused a considerable change in the kinetic parameters of contraction-relaxation cycles (**Fig. 3**, **4*B***). For instance, as compared to the contraction parameters under the effect of thapsigargin only, C-1087 caused a considerable increase (regarding the relevant parameters under the action of thapsigargin): the characteristic time of the relaxation phase (τ_C) – up to 130.7±4.1 % ($p < 0.01$, $n = 7$); the maximal velocity of the relaxation phase (V_R) – up to 135.3±4.6 % ($p < 0.01$, $n = 7$); force impulse at the maximal velocity level of the contraction phase (I_C) – up to 150.4±4.9 % ($p < 0.001$, $n = 7$).

Using ANOVA, we were able to establish that the percentage of influence of the factor (Ca^{2+}-pumps blockers: SERCA thapsigargin and PMCA thiacalix[4]arene C-1087) on the mechanokinetic parameters of spontaneous contractions of the myometrium is about 60–65 % (for example, for the amplitude it is 61 %), while the percentage of influence of other uncontrolled factors in the experiments is about 35–40 %.

Thus, under the conditions of SERCA blockage, further additional blocking of PMCA with thiacalix[4]arene C-1087 induced considerable changes in the mechanokinetics of contraction and relaxation phases. A considerable (by one-third) acceleration of the relaxation process is especially noteworthy. This controversial effect (regarding the effect of only C-1087 on V_R) may be caused by Ca^{2+}-regulated processes, including a probable increase in the outflow of K^+ ions via Ca^{2+}-sensitive K^+-channels (K_{Ca}) of PM which are essential regulators of myometrium relaxation (Brainard *et al*., 2007; Tsymbalyuk & Vadzyuk, 2020). Also, under the conditions of higher $[Ca^{2+}]_i$ level, especially in the near-membrane areas, there should be a considerable increase in the

activity of NO-synthases during PMCA blockage (Floyd & Wray, 2007; Pehlivanoğlu *et al*., 2013; Krebs, 2023); previously, we showed that the increase in NO concentration accelerated the process of myometrium relaxation (Kosterin *et al*., 2016).

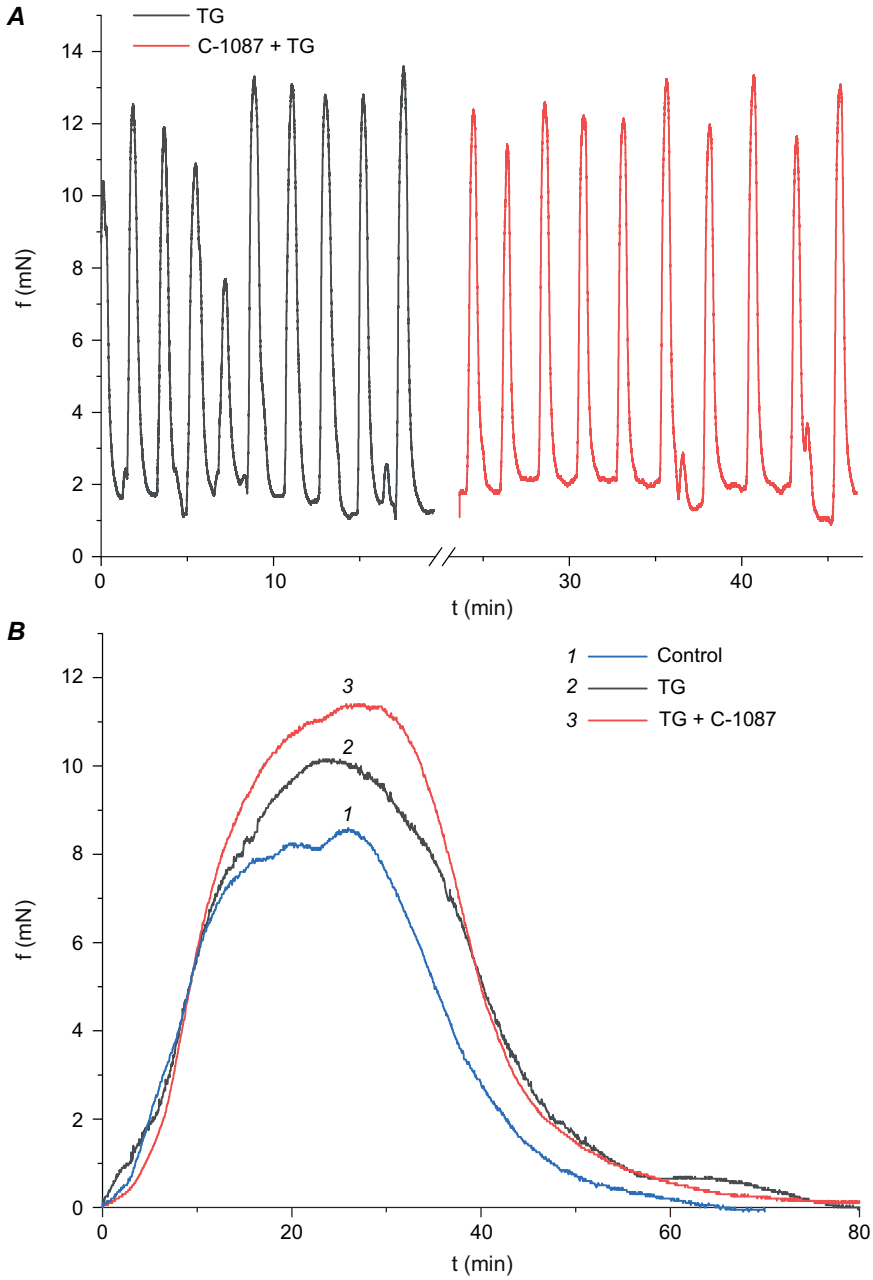


Fig. 4. The change in the spontaneous contractile activity in the rat myometrium under blocking of sarcoplasmic reticulum Ca^{2+}-pump with thapsigargin (TG, 0.5 μM) and the effect of thiacalix[4]arene C-1087 (10 μM) under the action of thapsigargin: ***A*** – a fragment of mechanograms; ***B*** – single spontaneous contractions

So our further studies were devoted to finding the effect of thiacalix[4]arene C-1087 and thapsigargin on the mechanokinetic effects regarding the spontaneous myometrium activity under preliminary blocking of NO-synthase activity.

Mechanokinetic effects of thiacalix[4]arene C-1087 under the conditions of blocking of SERCA and NO-synthases activity. The preliminary incubation of muscle preparations with the blocker of NO-synthases, L-NAME (100 μM, the duration of the preliminary incubation – 20 min), did not change the frequency-amplitude parameters of the spontaneous contractile activity (**Fig. 5**). However, taking these data into consideration, there was a noteworthy slowing-down of the contraction phase which was manifested by the increase in the values of the relevant mechanokinetic parameters (**Fig. 5**): the characteristic time of the contraction phase (τ_C) – up to 143.4±8.3 % ($p < 0.05$, $n = 7$); force impulse at the level of the maximal contraction velocity (I_C) – up to 157.8±9.9 % ($p < 0.01$, $n = 7$).

Under the condition of NO-synthases blocking, L-NAME (100 μM, the duration of the preliminary incubation – 20 min), SERCA blocker – thapsigargin (0.5 μM, the duration of the preliminary incubation – 20 min) caused a transient increase in the basal tension and a considerable increase in the amplitude and frequency of contractions (**Fig. 5*A***). There were also changes in some parameters of the contraction cycles, more considerable ones being present in the kinetics of the contraction phase (**Fig. 6**). As compared to the effect of L-NAME alone, under the effect of thapsigargin combined with the action of L-NAME, there was a considerable increase (in % regarding the effect of L-NAME, accepted as 100 %) in the following values: the time of reaching the amplitude (τ_0) – up to 155.0±3.9 % ($p < 0.01$, $n = 7$); the characteristic time of the contraction phase (τ_C) – up to 139.6±4.0 % ($p < 0.05$, $n = 7$); the characteristic time of the relaxation phase (τ_R) – up to 121.3±6.9 % ($p < 0.05$, $n = 7$); the force, at which the maximal velocity of the contraction phase is observed (F_C) – up to 120.5±4.0 % ($p < 0.05$, $n = 7$); the force impulse at the level of the maximal contraction velocity (I_C) – up to 169.8±15.4 % ($p < 0.01$, $n = 7$); the force impulse at the level of the maximal relaxation velocity (I_R) – up to 138.6±2.7 % ($p < 0.05$, $n = 7$); the force impulse at the level of the maximum (I_0) – up to 144.5±1.7 % ($p < 0.05$, $n = 7$).

Thus, SERCA blocking combined with NO synthesis blocking is accompanied by considerable changes in the mechanokinetics of the phases of contraction (which is manifested in the increase in the parameters τ_0, τ_C and I_C) and relaxation (which is manifested in the increase in the parameter I_R). However, when these results are compared with the parameters of spontaneous contractions under the effect of thapsigargin alone, the effects of nitric oxide synthases blocking are mostly related to the changes in the mechanokinetics of the contraction phase.

In the following series of experiments, we investigated the regularities of the effect of thiacalix[4]arene C-1087 in combination with blocking of NO-synthases and SERCA on the spontaneous contractile activity of the rat myometrium.

Thiacalix[4]arene C-1087 (10 μM) combined with SERCA blocking using thapsigargin (0.5 μM) and NOS non-selective blocker, L-NAME (0.1 mM), did not affect the amplitude-temporal characteristics of the spontaneous contractile activity of the myometrium, but caused a considerable modulation of the kinetics of some contractions both at the level of contraction and relaxation (**Fig. 5**, **6**). The relative values of kinetic parameters of spontaneous contractions under the effect of C-1087 combined with the action of L-NAME and thapsigargin (as compared to the relevant parameters under the

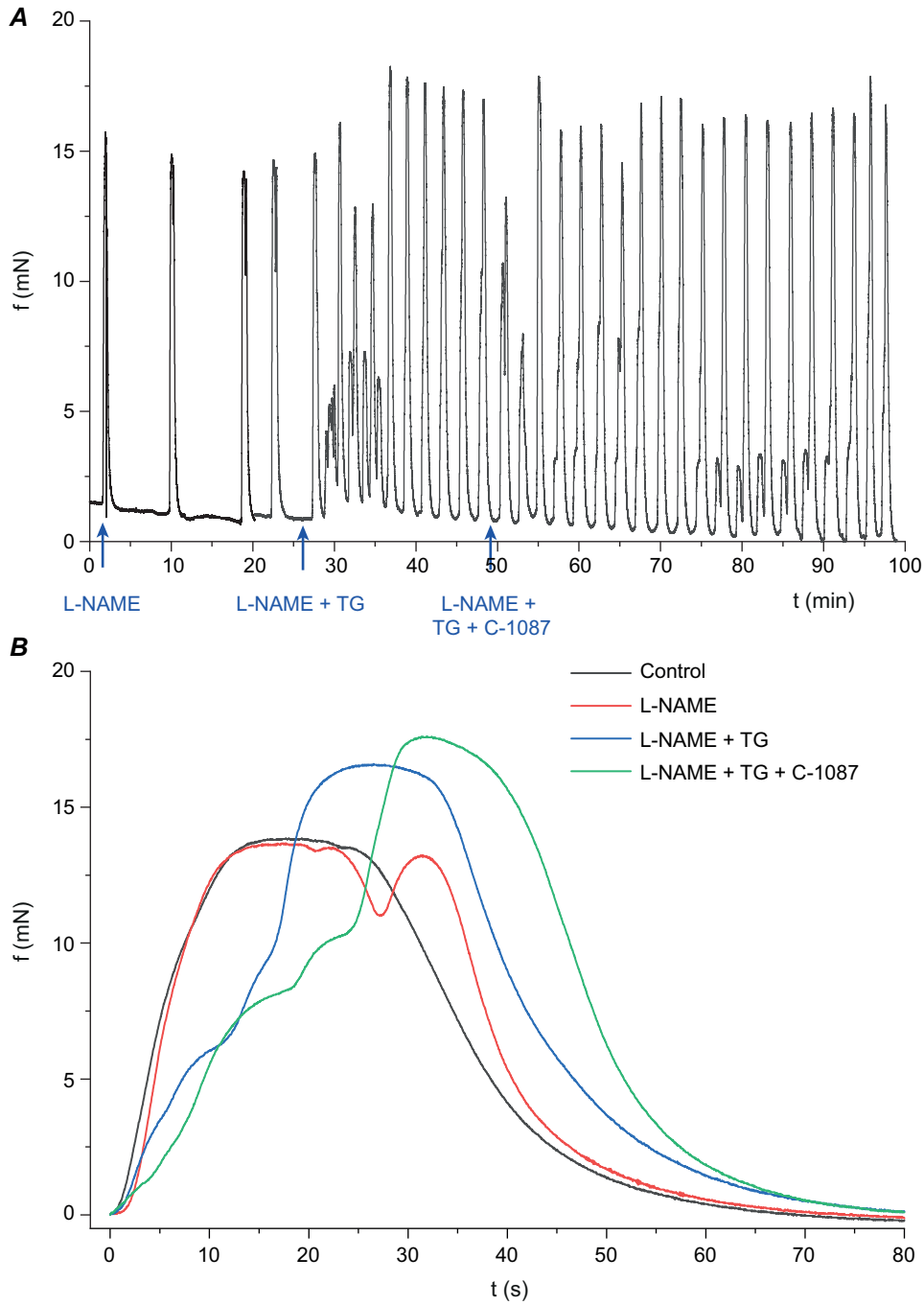


Fig. 5. The change in the spontaneous contractile activity in the rat myometrium under blocking of sarcoplasmic reticulum Ca^{2+}-pump with thapsigargin (TG, 0.5 μM) under the effect of the blocker of NO-synthases – L-NAME (100 μM), thiacalix[4]arene C-1087 (10 μM) under the combined action of thapsigargin and L-NAME: ***A*** – a fragment of mechanogram; ***B*** – single spontaneous contractions. Typical mechanograms are presented

effect of L-NAME or thapsigargin alone): the time of reaching the amplitude (τ_0) – up to 171.5 ± 12.2 % (p<0.01, n = 7); the characteristic time of the contraction phase (τ_C) – up to 219.8 ± 18.6 % (p<0.01, n = 7); the characteristic time of the relaxation phase (τ_R) – up to 155.2 ± 9.1 % (p<0.01, n = 7); the maximal velocity of the contraction phase (V_C) – up to 75.4 ± 5.6 % (p<0.05, n = 7); the maximal velocity of the relaxation phase (V_R) – up to 85.2 ± 1.9 % (p<0.05, n = 7); the force impulse at the level of the maximal contraction velocity (I_C) – up to 257.1 ± 20.8 % (p<0.001, n = 7); the force impulse at the level of the maximal relaxation velocity (I_R) – up to 160.7 ± 7.8 % (p<0.01, n = 7); the force impulse at the level of the maximum (I_R) – up to 146.9 ± 14.6 % (p<0.05, n = 7).

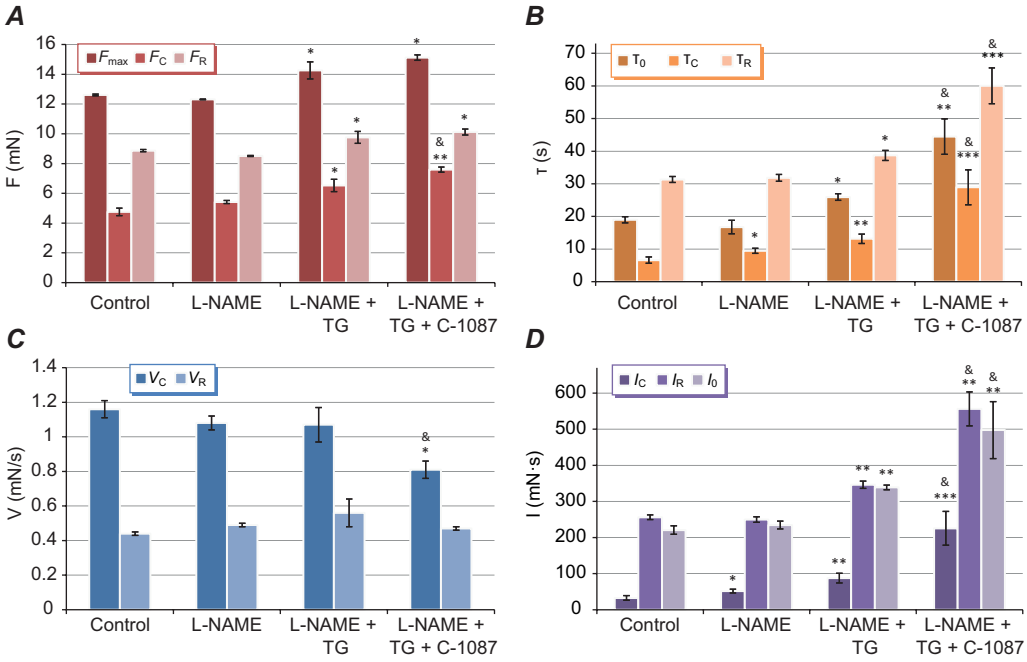


Fig. 6. The parameters of spontaneous contractions in the rat myometrium in control and under NOS blocking with non-selective blocker L-NAME (100 μM), SERCA – with thapsigargin (TG, 0.5μM), and the effect of thiacalix[4]arene C-1087 (10 μM) combined with thapsigargin. The parameters are: ***A*** – force (F_{max}, F_C and F_R); ***B*** – time (τ_0, τ_C and τ_R – respectively marked as tau 0, tau C, tau R); ***C*** – velocity (V_C and V_R); ***D*** – impulse (I_0, I_C and I_R). Data were presented as M ± SEM, n = 7. * – p <0.05; ** – p <0.01; *** – p <0.001 – compared with control; & – p <0.05 – compared with thapsigargin

Using ANOVA, we also established that the percentage of influence of Ca^{2+}-pump blockers (SERCA thapsigargin and PMCA thiacalix[4]arene C-1087) combined with NO-synthases blocking on the mechanokinetic parameters of spontaneous contractions of the myometrium increases and is about 70-80 %. In particular, in the case of amplitude parameters, this indicator was: for F_{max} 78.3 % (and for unaccounted factors 21.7 %), F_C 81.1 % (and for unaccounted factors 18.9 %) and for F_R 67.3 % (and for unaccounted factors 32.7 %). Therefore, the elimination of the contribution of NO-dependent changes in contractile activity makes it possible to significantly isolate the effects of blocking the primary active Ca^{2+} transport systems in myocytes, in particular, the contribution of PMCA to the regulation of spontaneous contractions of the myometrium.

Thus, the ability to inhibit the relaxation process by thiacalix[4] arene C-1087 (10 μM) under the preliminary blocking of SERCA is manifested during the blockade of NO-synthases. Since the effects of C-1087 combined with SERCA blocking in terms of spontaneous contraction parameters considerably depended on NOS activity, it is an additional confirmation of the inhibition of Ca^{2+}-pump of PM (which is a key regulator of NOS) under the effect of C-1087.

Mechanokinetic effects of thiacalix[4]arene C-1087 in combination with blocking of NO-synthases activity. A convenient pharmacological model for the study of the processes of SM contraction via the activation of Ca^{2+} inflow via the potential-governed channels and further extrusion of these cations during the relaxation phase is HPS-induced contraction (Trujillo *et al.*, 2000; Tsymbalyuk, 2018). It was shown in our previous studies (Veklich *at al.*, 2023) that under the conditions of preliminary incubation of SM preparations of the rat uterine with thiacalix[4]arene C-1087 (10 μM) there was a considerable slowdown in the process of relaxing the HPS-induced contraction and the index of the normalized maximal velocity of the relaxation phase decreased more than twice compared to the control. Thus the model of HPS-induced contraction is convenient for the investigation of the system of active transportation of Ca^{2+} ions via PM. In the following series of experiments, we investigated the regularities of the effect of thiacalix[4]arene C-1087 (10 μM) under the condition of preliminary NO-synthases blocking by L-NAME (0.1 mM, the duration of the preliminary incubation – 30 min).

The preliminary incubation of SM preparations with L-NAME (0.1 mM) caused a slight increase in HPS-induced contractions. The kinetic analysis demonstrated that blocking of NO-synthases was accompanied by some acceleration of the relaxation process (**Fig. 7**, mechanogram 2). In combination with L-NAME, thiacalix[4]arene C-1087 (10 μM) caused a complete inhibition of the relaxation process of the HPS-induced myometrium contraction which indicates a potent ability of this compound to block Ca^{2+} ion extrusion processes from SMC (**Fig. 7**, mechanogram 3).

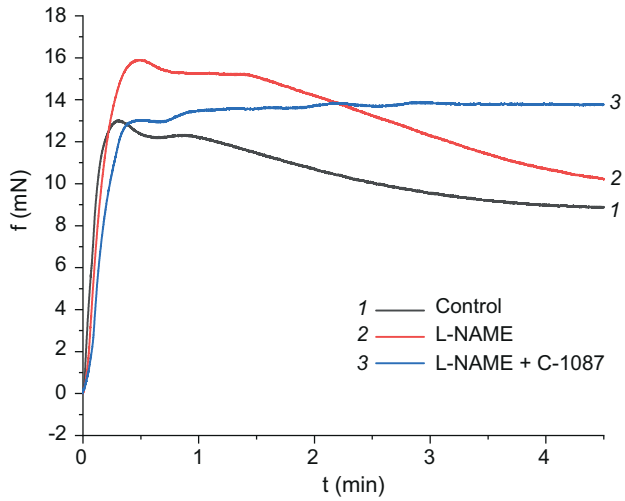


Fig. 7. The contraction of the myometrium of rats, induced by the application of the high-potassium solution (80 mM) in the control (*1*); under NO-synthases blocking by the preliminary incubation with L-NAME (0.1 mM, the duration of the preliminary incubation is 30 min) (*2*); under the effect of thiacalix[4]arene C-1087 (10 μM) combined with NO-synthases blocking by L-NAME (*3*). Typical mechanograms are presented

The effect of thiacalix[4]arene C-1087 on the intracellular concentration of Ca ions in the uterine myocytes in combination with SERCA blocking. In the following series of experiments, we investigated the effect of thiacalix[4]arene C-1087 under the condition of SERCA activity blocking on the intracellular concentration of Ca^{2+} in SMC of rat uterine.

The changes in the concentration of Ca^{2+} in myocytes under the effect of thiacalix[4] arene C-1087 were studied using the method of confocal microscopy and Ca^{2+}-sensitive probe fluo-4. Thapsigargin (0.1 μM) was added to the medium of myocytes incubation 15 minutes prior to the registration. We demonstrated that under the effect of thiacalix[4]arene C-1087 (20 μM), there was a sharp increase in the fluorescent response of Ca^{2+}-sensitive probe fluo-4 AM in the cell (**Fig. 8**, **9**). In the course of 2.5 min, the concentration of Ca^{2+} decreased, which demonstrated the involvement of the compensatory mechanisms (Ca^{2+}-uniporter of mitochondria, Na^{+}-Ca^{2+}-exchanger of PM) until the relaxation of the calcium signal. However, it did not return to the initial level but remained higher in the cell. So, thiacalix[4]arene C-1087 – the inhibitor of Ca^{2+}, Mg^{2+}-ATPase of PM – induced the increase in Ca^{2+} concentration in SMC.

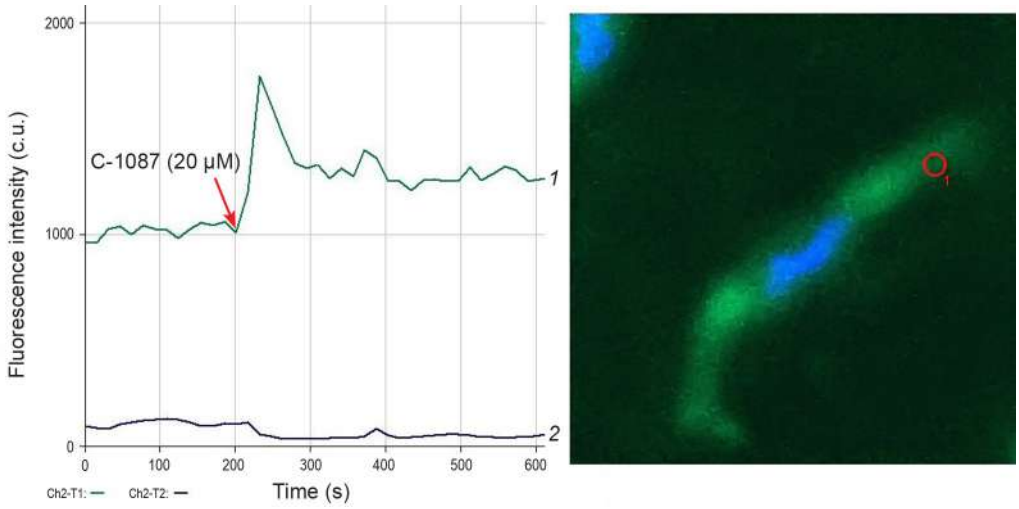


Fig. 8. The changes under the effect of thiacalix[4]arene C-1087 in the presence of thapsigargin (0.1 μM), the fluorescence of probes in the uterine myocyte which was registered using the confocal microscopy: Ca^{2+}-sensitive fluo-4 AM (*1*) and DNA-sensitive Hoechst (*2*). The introduction of the aliquot of the thiacalix[4]arene C-1087 solution (the final concentration – 20 μM) is indicated with the asterisks. The results of the typical experiment are presented

As shown by the simultaneous registration of the force of isometric contractions and Ca^{2+}-signal (Taggart & Wray, 1998), the change in the force in case of contractile activity and the change in the $[Ca^{2+}]_i$ profile in the myometrium of rats had similar kinetic characteristics. Under SERCA blocking with thapsigargin, thiacalyx[4]arene induced time-stable increase in $[Ca^{2+}]_i$ level and caused significant changes in myocyte functioning due to activation of Ca^{2+}-sensitive proteins, in particular the constitutive NO-synthases. Staged use of thapsigargin, L-NAME and C-1087 allowed us to clearly differentiate the contribution of PMCA to the development of the Ca^{2+} signal and maintenance of calcium homeostasis in uterine myocytes, as well as to demonstrate the kinetics of the inclusion

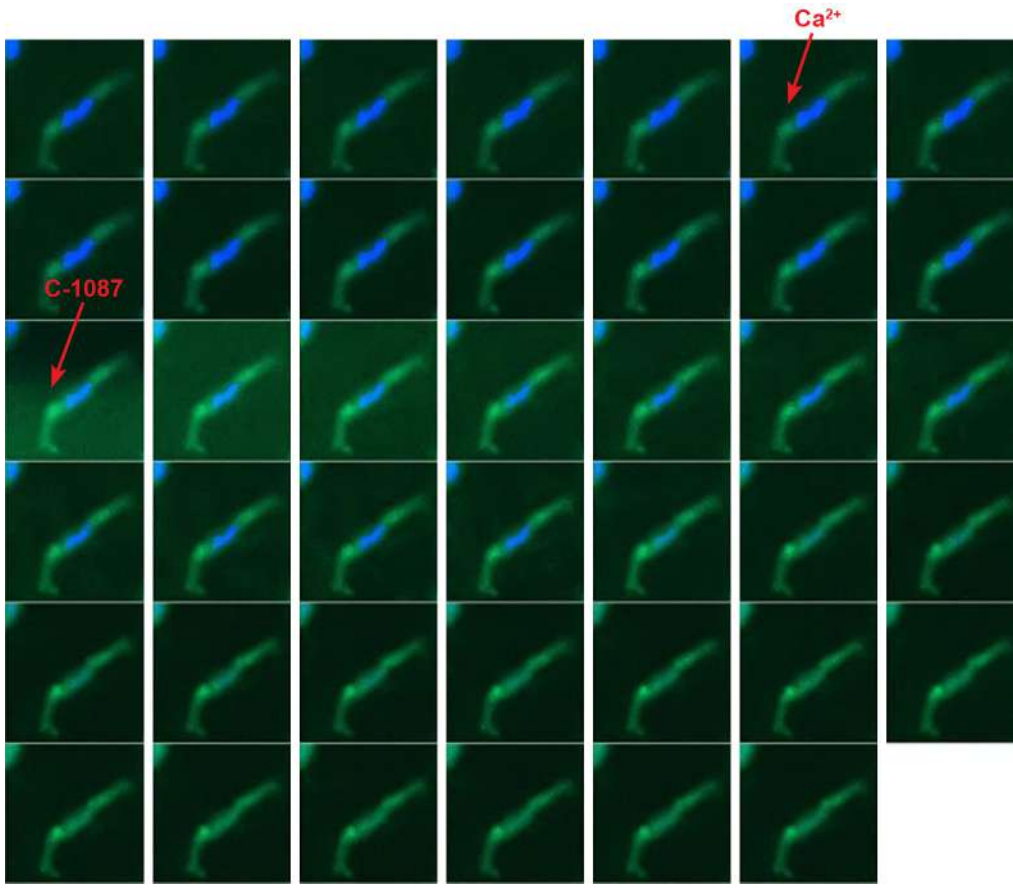


Fig. 9. The series of consecutive photographs of uterine myocytes using the scanning confocal microscope. Ca^{2+}-sensitive fluorescent probe fluo-4 AM. The arrows indicate the moments of introducing Ca^{2+} (1 μM) and thiacalix[4]arene C-1087 (20 μM). The medium for the incubation of myocytes contained thapsigargin (0.1 μM). The results of the typical experiment are presented

of other (non-SERCA and non-PMCA) transporting systems (probably, Ca^{2+}-uniporter of mitochondria and Na^+, Ca^{2+}-exchanger of PM (Matthew *et al.*, 2004; Floyd & Wray, 2007)) into the extrusion of Ca^{2+} from the cytosol of SMC which are only slightly capable of compensating the participation of PMCA and SERCA in the maintenance of basal level of $[Ca^{2+}]_i$.

CONCLUSIONS

The results of our studies demonstrate that while inhibiting PMCA (and probably SERCA, to some degree (Veklich *et al*., 2023)), thiacalix[4]arene C-1087 causes further changes in the functioning of Ca^{2+}-dependent systems in SMC. In general, it leads to changes in the kinetics of SM contraction-relaxation. While blocking the processes of active transportation of Ca^{2+} ions from the myoplasm, this compound slows the relaxation phase of both spontaneous and induced contractions of the myometrium considerably and also induces a spike in the fluorescent Ca^{2+}-signal of the isolated myocytes. However, PMCA-dependent mechanokinetic effects of C-1087 are considerably overlayed by the

secondary processes conditioned by the content of Ca^{2+} ions in the near-membrane area of SMC. In particular, it may be manifested in the permeability of K_{Ca}-channels and the activity of constitutive NO-synthases (Floyd & Wray, 2007; Pehlivanoğlu *et al*., 2013; Krebs, 2023). The latter assumption is well confirmed by the experiments studying HPS-activated contractions under the action of L-NAME. Therefore, it may be predicted that thiacalix[4]arene C-1087 is an efficient inhibitor of PMCA and a promising compound for the elaboration of pharmacological preparations on its basis to modulate the contractile activity in SM.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential 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.; V.K.; O.T.; T.V.]; methodology, [O.T.; T.V.; R.R.; S.K.; S.V.]; investigation, [O.T.; T.V.; R.R.; S.K.; S.V.]; data analysis, [O.T.; T.V.; R.R.; S.K.; S.V.]; writing – original draft preparation, [S.K.; V.K.; O.T.; T.V.; R.R.; S.K.; S.V.]; writing – review and editing, [S.K.; V.K.; O.T.; T.V.; R.R.; S.K.; S.V.]; visualization, [O.T.; T.V.; R.R.; S.K.; S.V.]; supervision, [S.K.; V.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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ТАПСИГАРГІН-РЕЗИСТЕНТНА ТІАКАЛІКС[4]АРЕН С-1087-ЧУТЛИВА СКЛАДОВА СКОРОТЛИВОЇ АКТИВНОСТІ МІОМЕТРІЯ ЩУРІВ ВІДДЗЕРКАЛЮЄ ФУНКЦІОНУВАННЯ КАЛЬЦІЄВОЇ ПОМПИ ПЛАЗМАТИЧНОЇ МЕМБРАНИ

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Обґрунтування. Відомо, що тіакалікс[4]арен С-1087 має високу здатність інгібувати Ca^{2+}-помпу (Ca^{2+},Mg^{2+}-АТФазу) плазматичної мембрани, однак він також певною мірою може пригнічувати функціонування Ca^{2+}-помпи саркоплазматичного ретикулуму гладеньких м'язів матки. Метою цієї роботи було вивчення ефектів С-1087 на скорочувальну активність і концентрацію іонів Ca^{2+} в клітинах міометрія щурів на тлі блокування Ca^{2+}-помпи саркоплазматичного ретикулуму відомим інгібітором цього ензиму тапсигаргіном.

Матеріали та методи. Експерименти проводили на безпородних білих щурах. Реєстрацію скорочувальної активності на препаратах поздовжньої СМ рогів матки зі збереженим ендотелієм проводили в ізометричному режимі. Для визначення змін $[Ca^{2+}]_i$ міоцити обробляли зондами Hoechst 33342 (для перевірки ядра клітини) та fluo-4 AM (для перевірки зміни концентрації Ca^{2+} у клітині).

Результати. Тензометричними дослідженнями в ізометричному режимі реєстрації з подальшим механокінетичним аналізом з'ясовано, що тіакалікс[4]арен С-1087 (10 мкМ) на тлі дії тапсигаргіну (0,5 мкМ) спричиняє суттєві зміни кінетики процесів спонтанної скорочувальної активності міометрія невагітних щурів, зокрема, зниження максимальної швидкості скорочення і зростання максимальної швидкості розслаблення. За допомогою конфокальної мікроскопії з використанням Ca^{2+}-чутливого флуоресцентного зонду fluo-4 встановлено, що аплікація тіакалікс[4]арену С-1087 до іммобілізованих міоцитів матки в умовах попередньої дії тапсигаргіну зумовлює транзієнтний сплеск Ca^{2+}-сигналу з подальшим виходом внутрішньоклітинної концентрації іонів Ca^{2+} на стабільно підвищений рівень. Ефекти С-1087 на тлі тапсигаргіну щодо фази розслаблення спонтанних скорочень міометрія усувались у разі попереднього блокування синтаз оксиду азоту L-NAME (100 мкМ). За попереднього аплікування L-NAME тіакалікс[4]арен С-1087 (10 мкМ) спричиняв повне пригнічення процесу розслаблення індукованого гіперкалієвим розчином (80 мМ) скорочення препаратів міометрія.

Висновки. Першопричиною змін скорочувальної активності і Ca^{2+}-сигналу в міоцитах матки за дії тіакалікс[4]арену С-1087 є його здатність пригнічувати Ca^{2+}-помпу плазматичної мембрани; подальші С-1087-індуковані зміни в тканині гладеньких м'язів, ймовірно, зумовлені підвищеним рівнем концентрації Ca^{2+} в міоцитах. Отримані результати вказують, що тіакалікс[4]арен С-1087 є перспективною сполукою для розробки на його основі фармакологічних препаратів для модуляції скорочувальної активності гладеньких м'язів, зокрема, міометрія.

Ключові слова: Ca^{2+},Mg^{2+}-АТФаза плазматичної мембрани, тіакалікс[4]арен С-1087, спонтанні скорочення, Ca^{2+}-сигнал

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