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THIACALIX[4]ARENE CHALCONE AMIDES EFFECT ON MYOMETRIUM CONTRACTION

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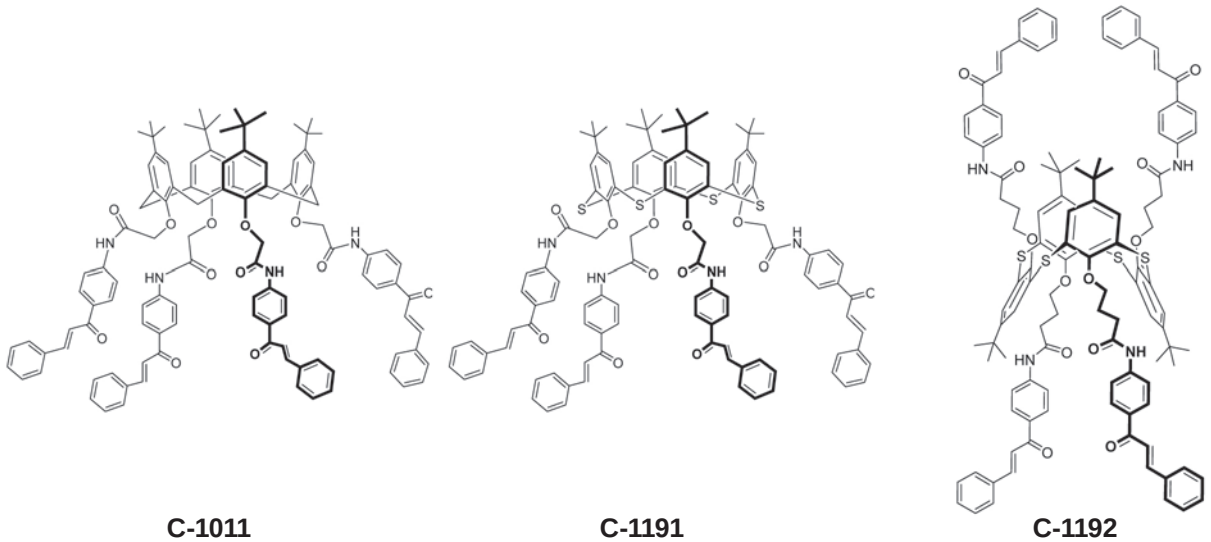
Calixarenes are macrocyclic compounds, the biochemical effects of which are actively studied. In this study we synthesized thiacalix[4]arene chalcone amides C-1191 and C-1192, which have a sulfur atom in their structure and different spatial arrangement of chalcone amide groups, and studied their effect on myometrium functioning. Experiments were conducted with the use of rat uterine smooth muscles preparations, isolated myometrial mitochondria and permeabilized myometrial cells. The relative value of mitochondria membrane potential (Δψ) was assayed with a voltage-sensitive fluorescent probe TMRM. The spontaneous contractive activity was studied by tenzometric method followed by mechanokinetic analysis. It was shown that C-1191 and C-1192 induced mitochondria hyperpolarization and increased the basal tension of myometrium smooth muscle preparation. Thiacalix[4]arene C-1191 did not change the uterine cycle, but increased the force, velocity and impulse parameters of muscle contractive activity. On the contrary, C-1192 modified the uterine cycle considerably, increased the total efficiency of the myometrium spontaneous contractive activity and decreased the force, time and impulse parameters. It is concluded that changes in the mechanokinetic parameters of myometrial contractile activity induced by C-1191 and C-1192 are determined by the functional activity of mitochondria.

K e y w o r d s: thiacalix[4]arene chalcone amides, mitochondria, mitochondria membrane potential, myometrial contractile activity, mechanokinetic analysis.

Calixarenes are macrocyclic compounds, the biological activity of which is determined by chemical groups at the upper and lower rims. In addition, it is possible to attach the different amounts of functionally active groups to the calix[4]arene macrocycle [1-5]. In previous works, we showed the effect of calix[4]arene chalcone amides on the mitochondrial membrane polarization. It was also shown that short-term incubation of myometrial mitochondria with calix[4]arene chalcone amides is accompanied by an increase in the average hydrodynamic diameter of mitochondria, which indirectly indicates the swelling of these organelles [6]. The effect of calix[4]arene chalcone amides on the hydrodynamic diameter of mitochondria increases with an increase in the number of chalcone residues in the calix[4]arene structure [6]. Using calix[4]arene chalcone amide C-1070 (a fluorescent analog of C-1011, Fig. 1) and confocal microscopy, it was proved that these compounds penetrate into myometrial cells [7]. The modulating effect of calix[4]arene chalcone amide (with two chalcone amide groups) on the polarization of mitochondrial membranes was shown on the primary myometrial cells culture using the potential-sensitive probe JC-1 [7].

For further investigation, we choose thiacalix[4]arene chalcone amides C-1191 and C-1192 (Fig. 1). Thiacalix[4]arene macrocycle is wider than calix[4]arene, so the distance between chalcone amide groups in C-1191 (conformation cone) is greater than in the calixarenes C-1011 and C-1070. Moreover, the conformational mobility of the thiacalixarene ring gives possibility to obtain macrocycle C-1192 in conformation 1,3-alternate where chalcone amide moieties are attached to macrocycle via longer γ–hydroxybutyramide linkers. Therefore, the dis-



C-1011 **C-1191** **C-1192**

Fig. 1. Structural formulas of calix[4]arene (C-1011) and thiacalix[4]arene (C-1191 and C-1192) chalcone amides

tance and spatial orientation of the chalcone amide groups in C-1192 are significantly different from C-1191 and other calix[4]arene chalcone amides that we have studied previously.

The main purpose of this work is to investigate the influence of structural changes in a series of calix[4]arene chalcone amide C-1011 and thiacalix[4]arene chalcone amides C-1191 and C-1192 on uterine contractions and the possible role of mitochondria in these effects.

Materials and Methods

The NMR spectra were registered on a Varian VXR 400 spectrometer operating at 400 MHz (1H), using TMS as a reference. The synthesis was carried out in dry solvents under atmosphere of dry nitrogen. TLC was performed with silica gel plates (Macherey-Nagel, AluGram, Xtra, G/UV254). Column chromatography was carried out with silica gel (S60, ROCC Belgium, 0.4 – 0.6 mm particle size).

Synthesis of thiacalix[4]arene chalcone amides (*general procedure*):

To a solution of acid **1** [8] or **3** [9] (0.50 g, 0.49 mmol) in dry methylene chloride (10 ml), oxalyl chloride (4 ml) and DMF (1 drop) were added, and the mixture was stirred at room temperature for 24 h. The solvent was evaporated in vacuo, and the corresponding acyl chloride **2** or **4** was isolated quantitatively.

A solution of acyl chloride **2** or **4** and 4-aminochalcone (0.50 g, 2.26 mmol) in dry toluene (10 ml) was stirred at reflux for 5 h. The progress of the reaction was followed by TLC ($CHCl_3$/MeOH, v/v 20:1). After cooling the reaction mixture, the precipitated product was filtered off, washed with toluene, dried *in vacuo* and purified by column chromatography ($CHCl_3$/MeOH, v/v 10:1).

Thiacalix[4]arene chalcone amides C-1191 and C-1192 were synthesized starting from corresponding acids **1** [8] (Fig. 2) and **3** [9] (Fig. 3). Conversion of these acids to corresponding carboxyl chlorides **2** and **4** was achieved by the action of oxalyl chloride (with an excess of 50%) at room temperature. Due to the high sensitivity of obtaining products to the humidity, they were used for the next step without purification and NMR characterization. The final stage of the synthesis was carried out in dry toluene by boiling the corresponding acyl chloride with two-fold excess of 4-aminochalcone.

Both compounds C-1191 and C-1192 have *tert*-butyl groups on the upper rim and four chalcone amide groups on the lower rim, which are attached to the thiacalix[4]arene cycle. The phenolic rings of thiacalix[4]arenes are connected via sulfur atoms -S- but not through methylene groups -CH_2- (as regular calix[4]arenes of the C-1011 type). Therefore, thiacalixarene cycles have a larger diameter, and it influences on the distances between chalcone groups: in structural analogs C-1011 and C-1191 the difference approx. 10%. In addition, the thiacalixarene macrocycle is more flexible, and the hinge movements of the phenol rings relatively to macrocyclic plane of

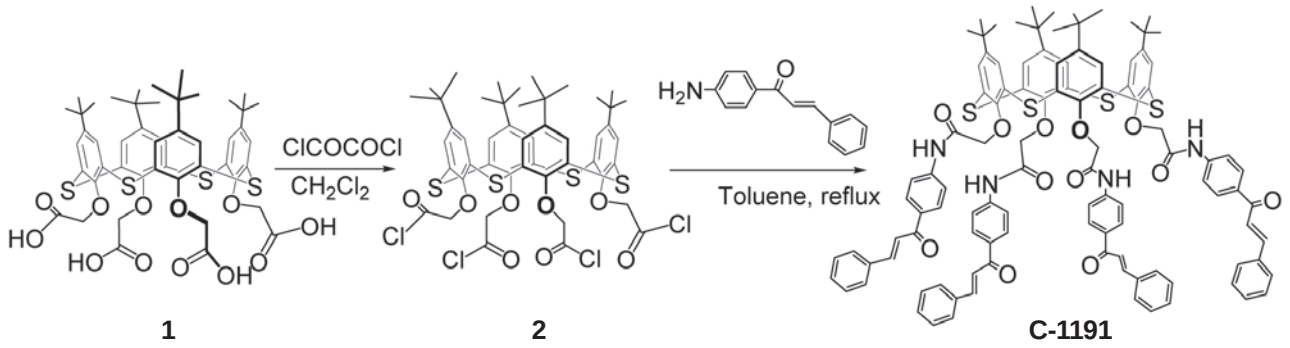


Fig. 2. Synthesis of 5,11,17,23-tetra-tert-butyl-25,26,27,28-tetra[(4'–benzylideneacetophenonyl)aminocarbonylmethoxy]-thiacalix[4]arene C-1191 (cone)

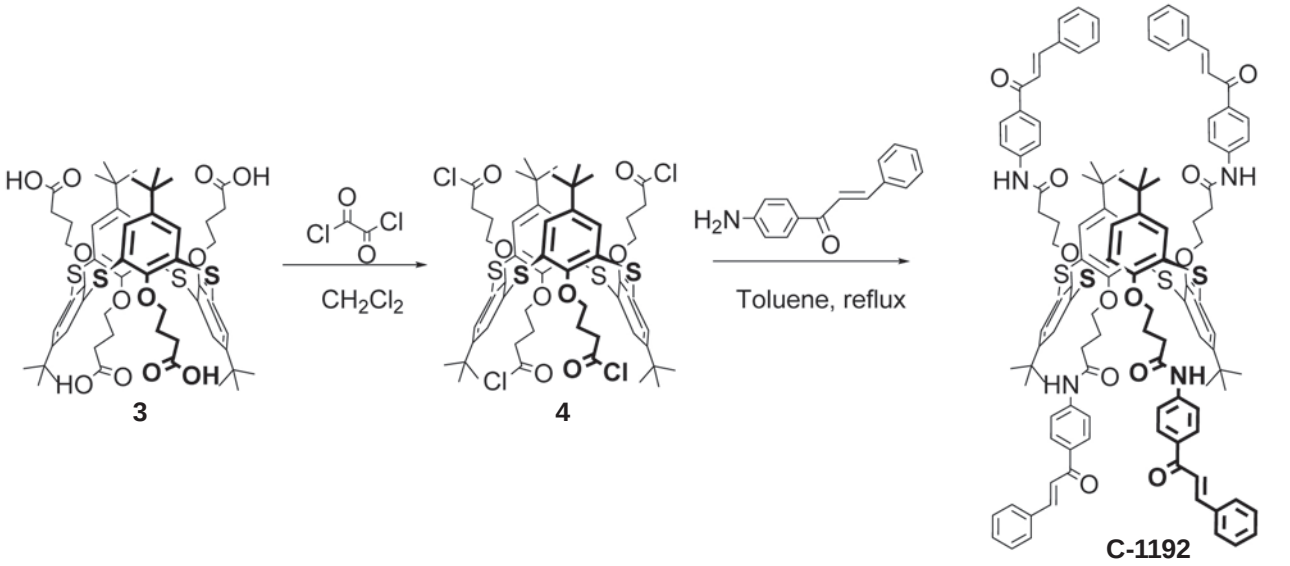


Fig. 3. Synthesis of 5,11,17,23-tetra-tert-butyl-25,26,27,28-tetra[(4'-benzylideneacetophenonyl)aminocarbonylpropoxy]-thiacalix[4]arene C-1192 (1,3-alternat)

the sulfur atoms have larger amplitude. On the one hand, this reduces the rigidity of the entire molecule, and so, it gives possibility to a better spatial arrangement of functional groups for cooperative interaction with biomolecules. The presence of sulfur atoms in the macrocycle can also influence the biological effects of such molecules due to coordination with metals and interaction with oxidants, such as peroxidases.

5,11,17,23-Tetra-*tert*-butyl-25,26,27,28-tetra[(4′-benzylideneacetopheno-nyl)aminocarbonylmethoxy)thiacalix[4]arene C-1191 (*cone*). Pale-yellow solid, R_f = 0.54, yield 65%, m.p. >300°C. ^{1}H NMR ($CDCl_3$), δ: 1.14 (s, 36H, C*Me*$_3$), 5.05 (s, 8H, -OC*H*$_2$), 7.32 (s, 8H, Ar*H*), 7.41 and 7.37 (m + d, 12H + 4H, Ar*H*), 7.52 (d, 4H, Ar*H*), 7.66 (m, 8H, Ar*H*), 7.75 (d, 4H, $^3J_{HH}$ = 8.4 Hz, CH=C*H*), 7.82 (d, 4H, $^3J_{HH}$ = 8.4 Hz, C*H*=CH), 9.68 (br. s, 4H, N*H*). Anal. Found: C 75.38%; H 5.94%; N 3.16%; S 7.25%. Calc. for $C_{108}H_{100}O_{12}N_4S_4$: C 75.14%; H 5.84%; N 3.25%; S 7.43%.

5,11,17,23-Tetra-*tert*-butyl-25,26,27,28-tetra[(4'-benzylideneacetopheno-nyl) aminocarbonylpropoxy]-thiacalix[4]arene C-1192 (*1,3-alternat*). Pale-yellow solid, R_f = 0.54, yield 57%, m.p. >300°C. ^{1}H NMR ($CDCl_3$), δ: 1.15 (s, 36H, C*Me*$_3$), 1.50 (t, 8H, -OCH$_2$C*H*$_2$), 2.21 (t, 8H, -C*H*$_2$C(O)), 3.94 (t, 8H, -OC*H*$_2$), 7.32 (s, 8H, Ar*H*), 7.41 and 7.37 (m + d, 12H + 4H, CH=C*H* +Ar*H*), 7.52 (d, 4H, Ar*H*), 7.66 (m, 8H, Ar*H*), 7.75 (d + m,

4H + 8H, C*H*=CH + Ar*H*), 7.82 (d, 8H, ArH), 9.68 (br. s, 4H, N*H*). Anal. Found: C 75.38%; H 5.94%; N 3.16%; S 7.25%. Calc. for $C_{116}H_{116}O_{12}N4S_4$: C 75.78%; H 6.36%; N 3.05%; S 6.97%.

Animals. The treatment of the lab animals was carried out according to "European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes" (Strasbourg, 1986) and the Law of Ukraine "On protection of animals from cruelty". The experimental protocol has been approved by the Institutional Bioethical Committee. Female non-pregnant rats were used. Experiments were performed using white female rats (weight 170÷240 g). Rats were kept under stationary vivarium conditions at constant temperature and basic allowance. Animals were narcotized with chloroform and then sacrificed using cervical dislocation.

Experiments were conducted on physiological models and two biochemical models (isolated myometrial mitochondria and permeabilized myometrial cells).

Isolation of myometrium mitochondria. Mitochondria from the myometrium of non-pregnant rats were isolated using the differential centrifugation method [10]. The mitochondria were suspended in a medium with the following composition: 250 mM sucrose, 1 mM EGTA, 20 mM Hepes, and buffered pH 7.4 at 4°C. Protein concentration of the mitochondrial fraction was determined by Bradford assay [11]. The concentration of mitochondrial protein in the sample was 25 μg/ml.

Isolation of myometrium cells. Myocyte suspension was isolated from non-pregnant rat myometrium with the collagenase method [12]. Isolated cells were suspended in Hank's solution (without salts of Mg and Ca) with the following composition: 136.9 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4, 4.5 mM $NaHCO_3$, 0.26 mM Na_2HPO_4, 5.5 mM glucose, 10 mM Hepes (pH 7.4). Calculation of cell amount was conducted using a hemocytometer. Test on the viability of isolated cells was made using trypan blue dye – over 95% of cells had green coloring, which proves the wholeness of the plasmatic membrane.

Dynamic light scattering. The function of mitochondria differentiation by the size was investigated with the dynamic light scattering method [13] using laser correlation spectrometer Malvern Instruments "ZetaSizer-3" (UK) with a He-Ne laser LGN-111 (P = 25 mW, λ = 663 nm). Registration of laser emission, dispersed (RI = 1.33) from mitochondria suspension, was made for 30 sec with a temperature 22°C and a dispersion angle 90°. The data was calculated using an application software PCS-Size mode v1.61.

Determination of ionized calcium concentration in the mitochondria matrix. $[Ca^{2+}]_m$ was determined using the QuantaMaster™ 40 spectrofluorometer (Photon Technology International) and the fluorescent probe Fluo-4, AM (λ_{ex}c = 490 nm, λ_{em} = 520 nm). Myometrium mitochondria were loaded with 2 μM Fluo-4, AM for 30 min at 37°C in a medium, the composition of which is indicated above. Thereafter, the suspension of mitochondria was diluted (1:10) in the same medium containing no fluorescence probe followed by centrifugation. The pellet was resuspended in the same medium containing no fluorescence probe. The $[Ca^{2+}]_m$ was measured in a medium containing: 250 mM sucrose, 2 mM K^+-phosphate buffer, 5 mM sodium succinate, 3 mM $MgCl_2$, ±3 mM ATP, ±0.1 mM $CaCl_2$, 20 mM Hepes, pH 7.4. To exclude the possibility of medium acidification by adenosine triphosphate addition, we normalized the pH of ATP stock solution with 1 M tris. The calibration of the Fluo-4 fluorescence was performed at the end of each testing probe by adding 0.1% Triton X-100 (in the presence of 100 μM $CaCl_2$) and, in 1 min, 5 mM EGTA (fluorescence intensities Fmax and Fmin, respectively). $[Ca^{2+}]_m$ was calculated using the Grynkiewicz equation [14].

Determination of ionized calcium concentration in the incubation medium. Ionized Ca concentration in the incubation medium ($[Ca^{2+}]_o$) was determined using the QuantaMasterTM 40 spectrofluorometer (Photon Technology International) and the fluorescent probe 1 μM Fluo-4, Pentapotassium Salt, cell impermeant (λ_{exc} = 490 nm, λ_{em} = 520 nm). The $[Ca^{2+}]_o$ was measured in a medium containing: 250 mM sucrose, 2 mM K^+-phosphate buffer, 5 mM sodium succinate, 3 mM $MgCl_2$, ±3 mM ATP, 20 mM Hepes, pH 7.4. To exclude the possibility of the medium acidification by adenosine triphosphate addition, we normalized the pH of ATP stock solution with 1 M tris. The calibration of the Fluo-4, Pentapotassium Salt fluorescence was performed at the end of each testing probe by adding 5 mM EGTA and, in 1 min, 5 mM $CaCl_2$ (fluorescence intensities F_{min} and F_{max}, respectively). $[Ca^{2+}]_o$ was calculated using the Grynkiewicz equation [14].

Fluo 5F, Pentapotassium salt, (λ_{exc} = 490 nm, λ_{em} = 520 nm) was used to test the possible effects of C-1191 and C-1192 on the fluorescence spectrum of the probe.

Measurements of mitochondria membrane polarization. To record mitochondria membrane polarization, a COULTER EPICS XL™ (Beckman Coulter, United States) flow cytometer with an argon laser (λ_{ex} 488 nm) was used. Experimental data was analyzed using the SYSTEM II™ Software (Beckman Coulter). Relative values of mitochondria membrane potential ($\Delta\psi$) were assayed using a voltage-sensitive fluorescent probe TMRM (Invitrogen) (λ_{ex} 488 nm, λ_{em} 590 nm) in the medium of the following composition: 20 mM HEPES (pH 7.4); 125 mM KCl; 25 mM NaCl; 2 mM K^+ phosphate buffer (pH 7.4); 5 mM sodium succinate; and 0.1 mg/ml digitonin (Merck). It should be noted that permeabilization of the plasma membrane with 0.01% digitonin excludes the contribution of its polarization to the probe signal. Cells suspension ($2–2.5\cdot10^5$ cells/ml) were incubated with calix[4]arene for 5 min at 37°C followed by loading with 100 nM TMRM and then immediately analyzed on the flow cytometer at a wavelength of 590 nm (channel FL_2). Each measurement was represented as the average fluorescent intensity of 10 000 events and expressed in relative units: average fluorescence intensity value of a sample minus average autofluorescence intensity.

Tenzometric experiments. The contractile activity of smooth muscles of rat uterine horns was studied using the preparations, in which the uterine horns were cut into 2×10 mm stripes in the longitudinal direction, leaving the endothelial layer intact since the myometrium tissue on the side of the ovaries had higher pacemaker activity, the horn fragments from this area were preferred.

All the preparative procedures were conducted in the Krebs physiological 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). Thiacalix[4]arenes C-1191 and C-1192 were dissolved in a non-polar solvent, DMSO; both substances were administered to the Krebs solution to achieve the final concentration of 10^{-5} M (DMSO concentration in the solution was 0.1%); the control contractions were registered on the background of 0.1% DMSO.

The spontaneous contractile activity was studied by tenzometric methods in the isometric mode. The preparations of smooth muscles were placed into the working chamber (efficient volume of 2.0 ml) of tenzometric equipment with the flowing Krebs solution (the flow rate of 5 ml/min) and left for one hour until achieving stable reproduction of contractions. The working chamber was thermostated using the liquid thermostat at 37.5 ± 0.3°C.

Mechanokinetic analysis of spontaneous contractions. he total efficiency of the spontaneous contractile activity in smooth muscles was evaluated by the following indices: the duration of specific cycles of spontaneous contractions, the pauses between them, the duration of specific fragments of contractions (contraction and relaxation phases), the asymmetry coefficient (the ratio between the duration of the contraction phase and the duration of the relaxation phase), the contractile cycle (the duration of a spontaneous contraction cycle and a pause thereafter), the index of activity for contractions (the ratio between the duration of the contraction cycles and the duration of pauses between them). The changes in the efficiency of cumulative contractile activity in smooth muscle preparations were quantitatively evaluated in Montevideo units for contraction indices (MU, the product of the mean amplitude and frequency of contractions in 10 min) and Alexandria units (AU, the product of MU and the mean duration of contractions in 10 min) [15].

The mechanokinetics of the spontaneous contractile activity in SM preparations was studied according to the method of multiparameter mechanokinetic analysis [16]. The analysis of the complete profile of single spontaneous contractions was based on the procedure of their linearization in the coordinates $[\ln(f_R/f_C);\ln(1+\Delta t/t)]$ (where f and t – instant values of force and time at the level of the contraction cycle, and indices C and R are symbols for the phases of contraction and relaxation, respectively). Hereinafter, F_C and F_R – the relevant values of the force at the inflexion points of the mechanogram at the level of the phases of contraction (from the beginning of the increase in the force to its maximal value F_{max}) and relaxation (from the maximal value of the force F_{max} at the time moment τ_0 and until its return to the basal level), Δt – arbitrarily fixed time interval.

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 – the maximal velocities of the contraction and relaxation phases, respectively, and I_0, I_C and I_R – force impulse parameters at the level of the amplitude and maximal velocities of the contraction and relaxation phases, respectively.

Statistical analysis of the data. The experimental data were processed using variation statistics methods in 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 t-test was used to determine the reliable differences between the mean values of two samples; the one-way ANOVA with Tukey post-hoc test to determine the reliable differences between the mean values of four groups (control and compounds C-1191, C-1192 and C-1011). The results were considered reliable on condition of the probability value P under 5% ($P < 0.05$). The validation analysis of data approximation by the linear function (linearization) was performed using Fisher's F-criterion; determination coefficients (R^2) were at least 0.96 in all cases. Results are reported as means ± SEM of 3-6 independent experiments (biological replicates).

Results and Discussion

The compounds C-1191 and C-1192 differ in conformation and length of the connecting chalcone amide groups methylene chain. In the molecule of C-1191, the thiacalixarene cycle adopts a *cone* conformation and the chalcone amide groups are connected to it by one methylene group (CH_2). Therefore, all chalcone fragments are located close to each other. (The structural analog of this compound is previously described calix[4]arene C-1011). In the molecule of C-1192, the thiacalixarene cycle is in the *1,3-alternate* conformation. Therefore, one pair of distal chalcone amide groups is located in one direction from the macrocyclic plane, the other – in the opposite direction. In addition, chalcone amide groups are connected to it through three methylene groups (CH_2-CH_2-CH_2), and are located quite far from each other and from the thiacalixarene cycle.

Using the method of dynamic light scattering, the hydrodynamic diameter of mitochondria was determined in the presence of thiacalix[4]arene chalcone amides C-1191 and C-1192 in concentrations of 1 and 10 μM. The obtained results prove that short-term incubation of mitochondria with thiacalix[4]arene chalcone amides was not accompanied by changes in the size of mitochondria, with the exception of 10 μM calix[4]arene C-1192, which increases the hydrodynamic diameter of mitochondria by an average of 25% compared to the control (Fig. 4).

It should be mentioned that calix[4]arene chalcone amide C-1011, which contains 4 chalcone groups and does not contain sulfur, significantly increased the hydrodynamic diameter of mitochondria as we showed previously [6]. Thus, thiacalix[4]arene chalcone amides have a less powerful effect on the size of mitochondria compared to calix[4]arene chalcone amides.

Next step, the effects of thiacalix[4]arene chalcone amides C-1191 and C-1192 on Ca^{2+} exchange in mitochondria were studied: 1) on the basal Ca^{2+}

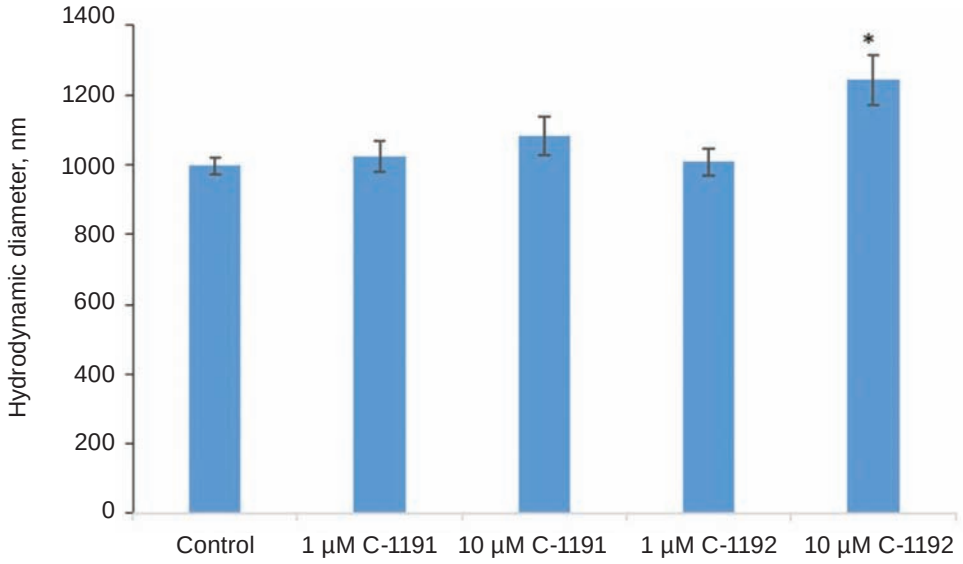


*Fig. 4. Effect of thiacalix[4]arene chalcone amides C-1191 and C-1192 on the hydrodynamic diameter of rat myometrial mitochondria. M ± m, n= 5. *P < 0.05 compared to the control*

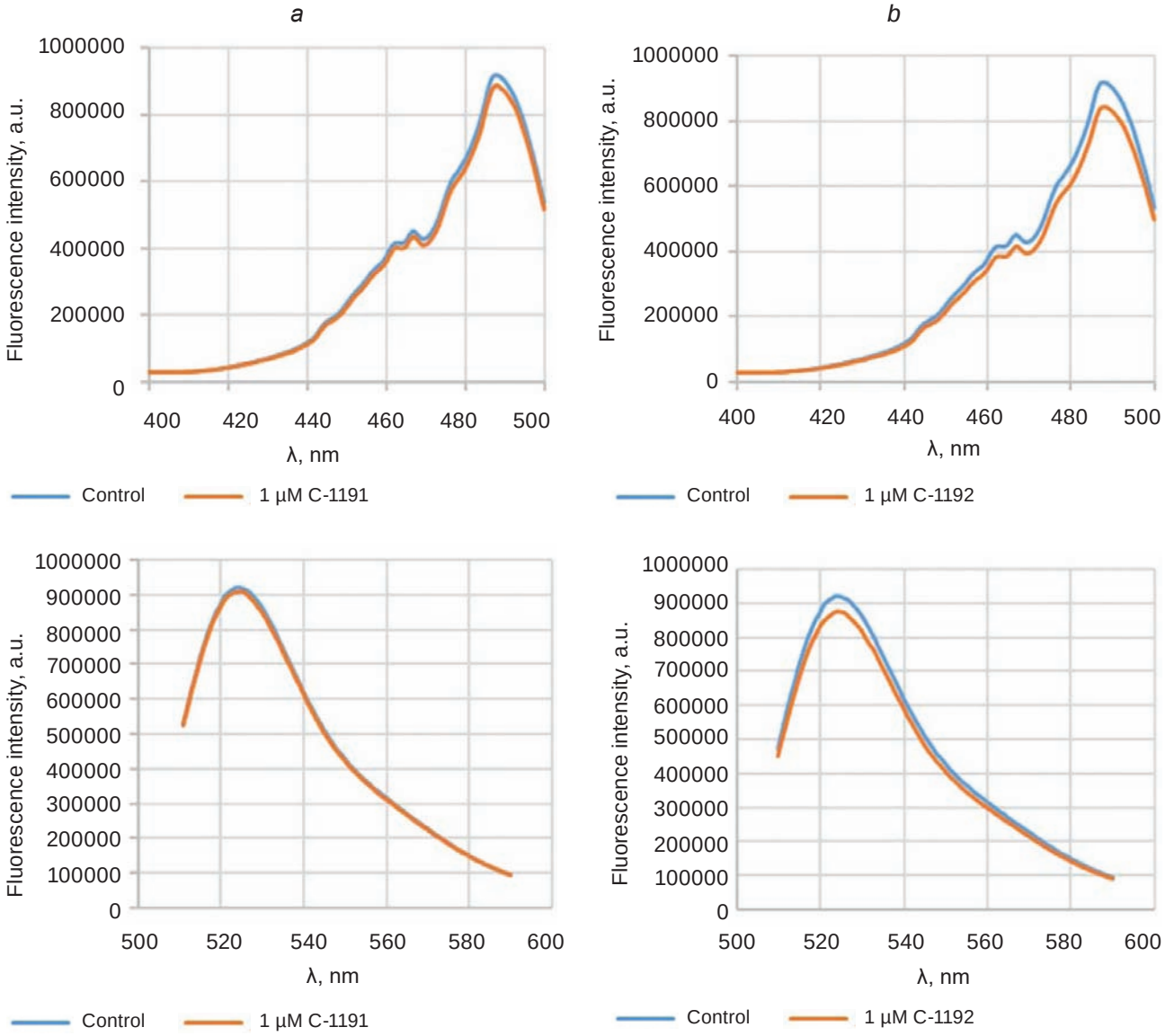


*Fig. 5. Absorption (λ_{em} = 520 nm) and fluorescence (λ_{ex} = 490 nm) spectra of 1 μM Fluo 5F, pentapotassium salt, (cell impermeant) in the presence of thiacalix[4]arene chalcone amides C-1191 (**a**) and C-1192 (**b**). The results of a typical experiment are presented (n = 4)*

concentration in the matrix and on the cation accumulation; 2) on the Ca^{2+} efflux from mitochondria. These experiments were started with clarifying the question of whether thiacalix[4]arene chalcone amides C-1191 and C-1192 affect the fluorescence of the Ca^{2+}-sensitive probe Fluo-5F, Pentapotassium Salt (a water-soluble analog of Fluo-4AM) (Fig.5).

As can be seen, 1 μM thiacalix[4]arene chalcone amides C-1191 and C-1192 did not affect the absorption and fluorescence spectra of the probe (Fig. 5).

Using the Ca^{2+}-sensitive fluorescent probe fluo 4AM, it was shown that 1 μM thiacalix[4]arene chalcone amides C-1191 and C-1192 did not affect both the basal Ca^{2+} concentration and the accumulation of this cation in the matrix of mitochondria (data not shown).

Using the Ca^{2+}-sensitive fluorescent probe Fluo-4, pentapotassium salt, it was shown that 1 μM thiacalix[4]arene chalcone amides C-1191 and C-1192 did not affect the Ca^{2+} efflux from mitochondria (data not shown).

Therefore, short-term incubation (5 min) of mitochondria in the presence of the studied thiacalix[4]arene chalcone amides is not accompanied by changes in Ca^{2+} concentration both in the organelle

matrix and in the incubation medium. It should be noted that short-term incubation of mitochondria with calix[4]arene chalcone amide C-1011 also did not have a significant effect on Ca exchange in mitochondria [6].

Further studies – testing the mitochondrial membranes polarization in the presence of thiacalix[4]arene chalcone amides. Using a suspension of myometrial cells treated with a digitonin solution, flow cytometry and a potential-sensitive TMRM probe, it was shown that 1 μM thiacalix[4]arene chalcone amides C-1191 and C-1192 increased the level of mitochondrial membrane polarization (Fig. 6).

Therefore, incubation of digitonin-permeabilized rat myometrial cells with 1 μM C-1191 or 1 μM C-1192 increased the fluorescence intensity of TMRM compared to the control, indicating an increase in mitochondrial membrane polarization. Calix[4]arene chalcone amide C-1011 had a similar effect on the polarization of mitochondrial membranes, as we have previously shown [7].

Since thiacalix[4]arene chalcone amides C-1191 and C-1192 changed the polarization of the internal membrane of mitochondria, it could also reflect on the functioning of the integral smooth muscle cells and their groups. Thus, in the next stage, we investigated the spontaneous contractile activity of pluricellular preparations of myometrium under the conditions of applying these substances in the concentration of 10^{-5} M.

It was determined that the administration of thiacalix[4]arene chalcone amide C-1191 into the Krebs solution, washing the smooth muscle preparations, caused the increase in their basal tension on average 10–12 min after the beginning of the substance effect (Fig. 7). On the background of the increased basal tension, there was a rise in the amplitude of the spontaneous contractions (on average up to 125.2 ± 3.9% (n = 5, P < 0.05 as compared to the control, accepted as 100%) without any significant impact on their frequency.

Noteworthy is the disruption of the uterine cycle under the effect of C-1191, so we analyzed the total efficiency of spontaneous contractions in myometrium 15 min after the start of the muscle preparation incubation with this thiacalix[4]arene chalcone amide. It was determined that on the background of C-1191, the duration of the contraction phase remained on the control level, whereas the duration of the relaxation phase increased considerably, on average up to 115.9 ± 5.5% (n = 5, P < 0.05); if the relaxation process was prolonged, there was a reliable increase in the total duration of spontaneous contraction-relaxation cycles (on average up to

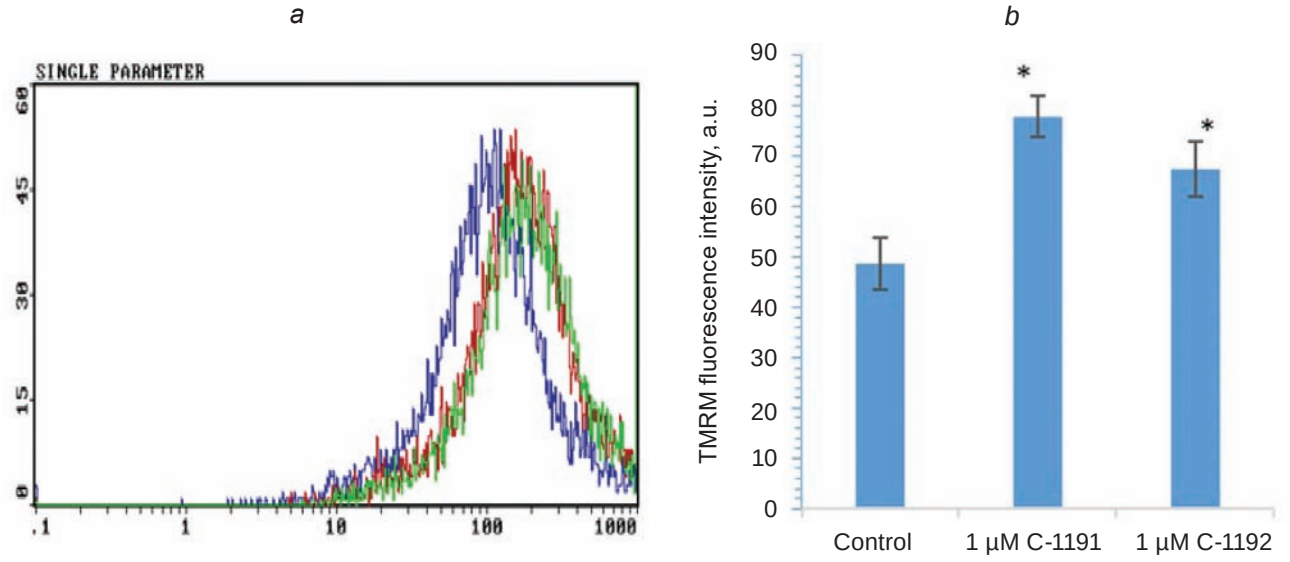


Fig. 6. Polarization of mitochondrial membranes. Incubation of permeabilized rat myometrial cells with 1 μM C-1191 or 1 μM C-1192: ***a****) blue graph – control, red graph – 1 μM C-1191, green graph – 1 μM C-1192;* ***b****) each measurement was expressed in relative units: average TMRM fluorescence intensity value of a sample minus average autofluorescence intensity (M ± m, n = 5, P < 0.05 compared to control). Fig 6.a - the results of a typical experiment are presented (n = 5)*

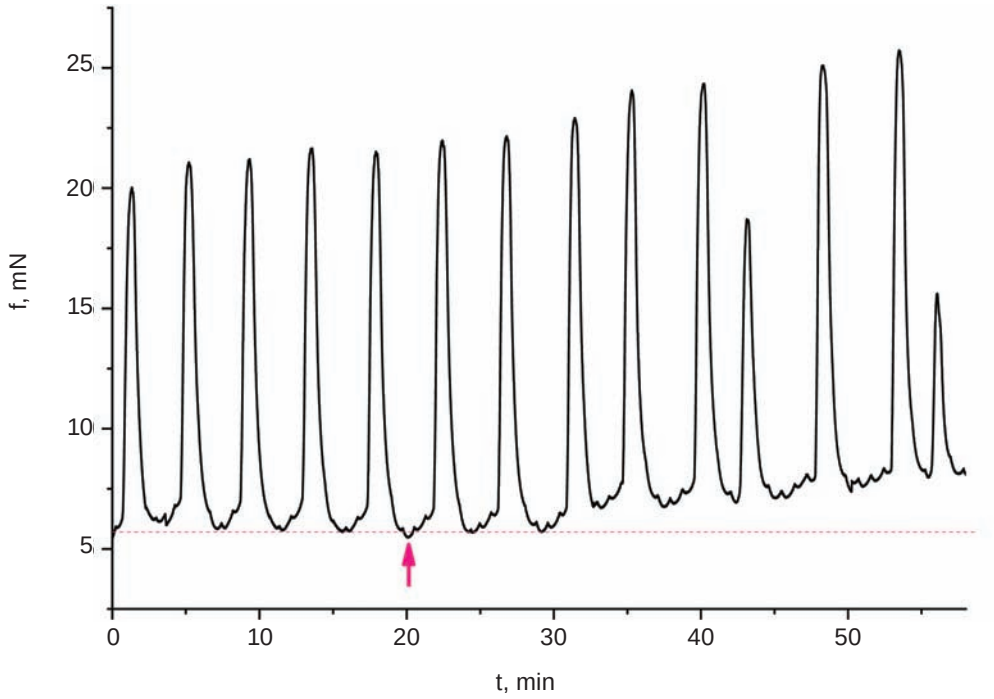


Fig. 7. The typical mechanogram of the modulation of spontaneous contractions in the longitudinal smooth muscles of rat uterine horns and under the effect of thiacalix[4]arene chalcone amide C-1191 (10 μM). A typical mechanogram is presented. The basal tension of the preparations is indicated with the dashed line. The moment of adding C-1191 to the normal Krebs solution is shown with the asterisk

117.2 ± 6.3%, $n = 5$, $P < 0.05$). Also, due to a combined increase in the amplitude of spontaneous contractions and the duration of the contraction-relaxation cycle, there was a considerable enlargement of the area under some spontaneous contraction-relaxation cycles (on average up to 120.9 ± 2.1%, $n = 5$, $P < 0.05$). The indices of the total efficiency of the spontaneous contractile activity in the myometrium were at the control level too – the contraction indices in MU units (on the background of 10-5 M thiacalix[4]arene chalcone amide C-1191 it was 105.4 ± 7.4, $n = 5$, $P > 0.05$, on average) and in the AU (under the effect of C-1191, it was 114.0± 6.9, $n = 5$, $P > 0.05$, on average).

Further on, to predict possible targets of the thiacalix[4]arene chalcone amide C-1191 effect on the myometrium tissue, some contraction-relaxation cycles were analyzed by the method of multiparameter mechanokinetic analysis [16]. It was found that the C-1191 caused a considerable and even increase in the force parameters F_C (on average up to 127.5 ± 4.5% as compared to the control, $n = 5$, $P < 0.05$) and F_R (on average up to 125.2 ± 2.3% as compared to the control, $n = 5$, $P < 0.05$) (Fig. 8, *A*). Thiacalix[4]arene chalcone amide C-1191 did not affect the time parameters (τ_0, τ_C and τ_R) of the spontaneous contractions (Fig. 8, *B*), yet reliably increased the indices of maximal velocities of the contraction and relaxation phases of the spontaneous cycles: on the level of the contraction phase V_C on average up to 123.7 ± 1.9 and V_R on average up to 118.7 ± 4.3%, in both cases $n = 5$, $P < 0.05$ (Fig. 8, *C*). However, the norm-setting for maximal velocities with the consideration of the contraction amplitude eliminated the differences in the velocities of the contraction and relaxation phases regarding the relevant control parameters, so the specific impact of the C-1191 compound on the processes of the uptake and extrusion of Ca^{2+} ions of myocytes is likely to be absent. The evaluation of impulse parameters (I_0, I_C and I_R) demonstrated a reliable increase in the force impulse under the amplitude value (on average up to 127.1 ± 2.8%, $n = 5$, $P < 0.05$) and in the relaxation phase (on average up to 125.9 ± 3.5%, $n = 5$, $P < 0.05$), while the force impulse in the contraction phase remained at the control level (Fig. 8, *D*).

Thus, thiacalix[4]arene chalcone amide C-1191 induced an even increase in all the force (F_{max}, F_C and F_R), velocity (V_C and V_R), and some impulse parameters (I_0 and I_R); so the effects of C-1191 cannot

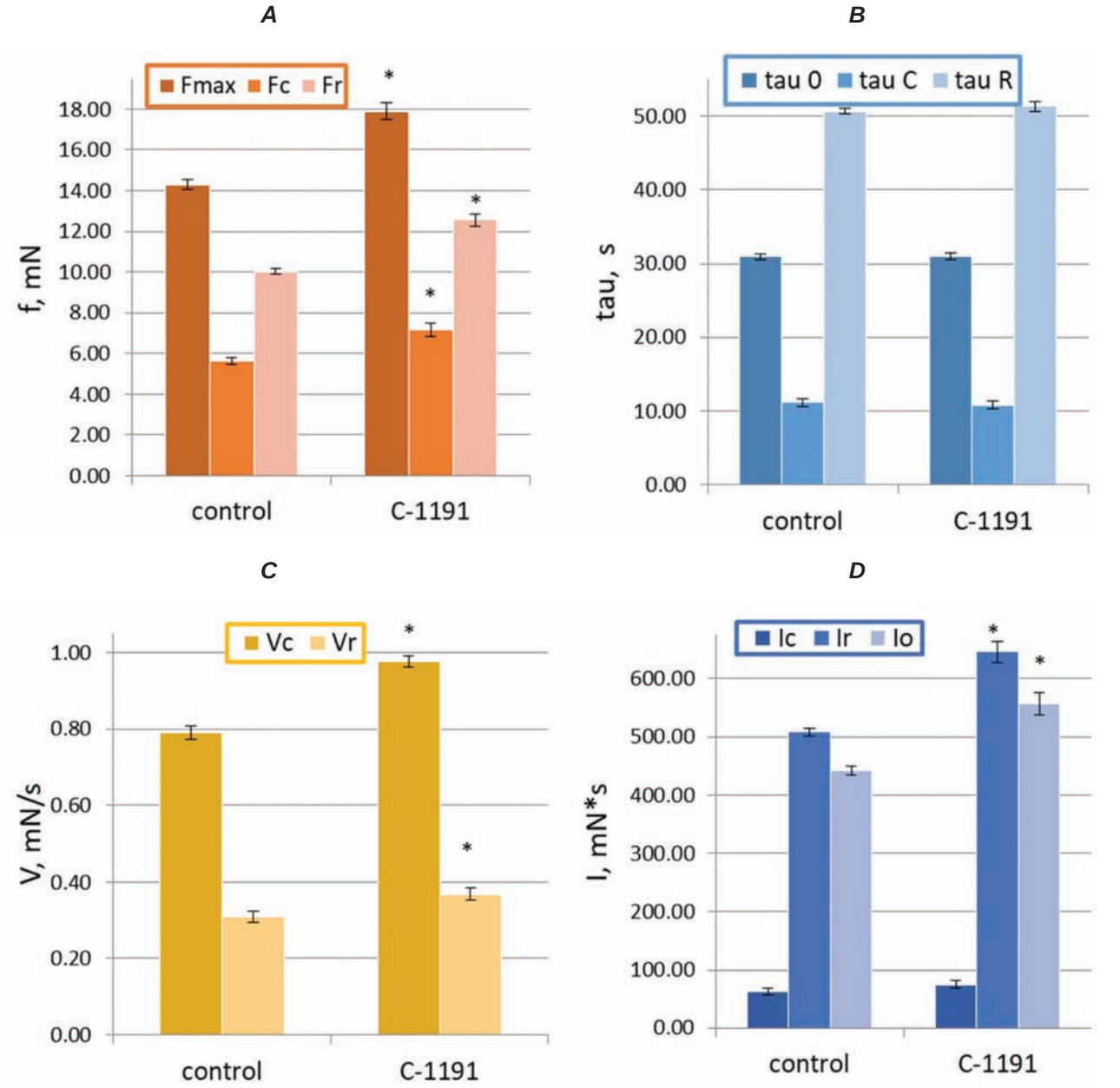


*Fig. 8. The mechanokinetic parameters of the spontaneous contractile activity of rat myometrium in the control and under the previous incubation on the background of thiacalix[4]arene chalcone amide C-1191 (10 μM): **A** – force parameters (F_{max}, F_C and F_R); **B** – time parameters (τ_0, τ_C and 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 – significant against control*

be considered specific in terms of some systems, involved in the realization of the cellular mechanisms of the contraction-relaxation cycle.

In the next stage, we investigated the spontaneous contractile activity of pluricellular preparations of myometrium under the conditions of applying thiacalix[4]arene chalcone amide C-1192 (10^{-5} M). Similar to the C-1191 compound, the addition of thiacalix[4]arene chalcone amide C-1192 to the solution, washing the smooth muscle cells, was accompanied by the rise in their basal tension (Fig. 9). However, contrary to the previously studied compound, on the background of C-1192, there was a considerable decrease in the amplitude of contractions (on average down to 78.1 ± 5.7%, $n = 5$, $P < 0.05$, as compared to the control, accepted as 100 %) on the background of

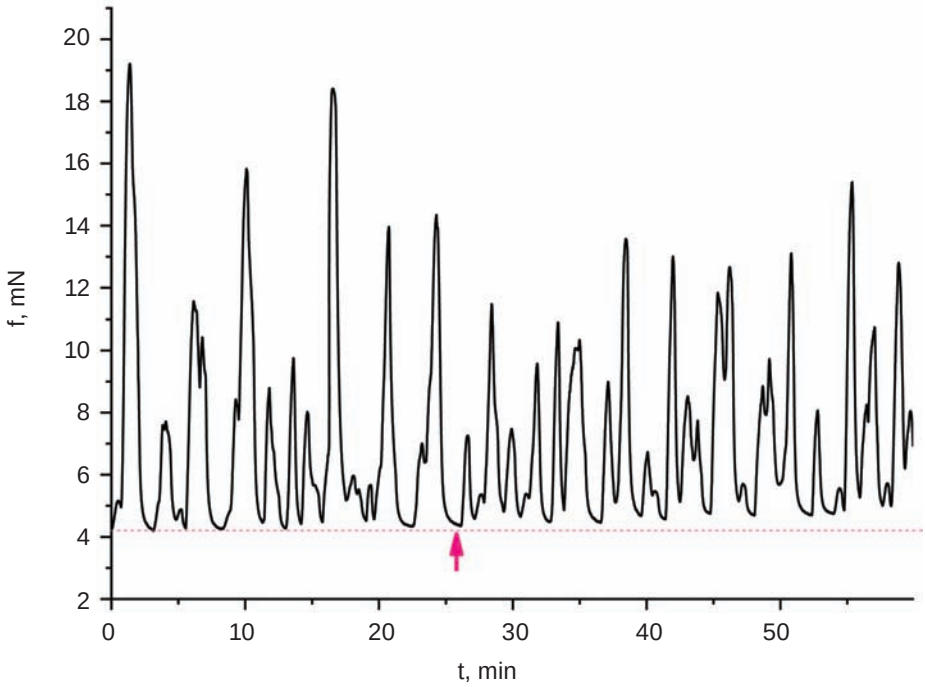


Fig. 9. The typical mechanogram of the modulation of spontaneous contractions in the longitudinal smooth muscles of rat uterine horns and under the effect of thiacalix[4]arene chalcone amide C-1192 (10 μM). The basal tension of the preparations is indicated with the dashed line. The moment of adding C-1192 to the normal Krebs solution is shown with the asterisk

a significant increase in their frequency (on average up to 172.4 ± 8.3%, $n = 5$, $P < 0.05$, as compared to the control, accepted as 100%).

Then, the total efficiency of the uterine cycle under the effect of the C-1192 compound was studied. It was found that on the background of this thiacalix[4]arene chalcone amide (contrary to C-1191), the duration of the relaxation phase tended to decrease and was downsized to 82.1 ± 14.7% ($n = 5$, $P > 0.05$ as compared to the control) on average; the duration of the contraction phase decreased considerably (on average down to 66.2 ± 6.0% ($n = 5$, $P < 0.01$ as compared to the control). In addition, the effect of C-1192 induced a considerable decrease in the total duration of the uterine cycles of contraction-relaxation (on average, down to 43.4 ± 6.1%, $n = 5$, $P < 0.001$ as compared to the control). Under these conditions, the duration of pauses between contractions was, on average, 13.1 ± 7.2%, $n = 5$, $P < 0.001$ as compared to the control. Also, the area under some spontaneous contraction-relaxation cycles decreased twice (down to 49.1 ± 10.7%, $n = 5$, $P < 0.001$ as compared to the control).

Generally, thiacalix[4]arene chalcone amide C-1192 induced a considerable increase in the total efficiency of the spontaneous contractile activity of myometrium, which was reflected in quantitative terms in the increase of the MU contraction indexes (on average up to 204.9 ± 22.6, $n = 5$, $P < 0.001$ as compared to the control) and in AU indexes (on average up to 152.6 ± 14.1, $n = 5$, $P < 0.001$ as compared to the control).

Later, we analyzed some contraction-relaxation cycles using the multiparameter mechanokinetic analysis [16]. It was found that the C-1192 caused a considerable decrease in the force parameters F_C (on average down to 79.4 ± 4.8% as compared to the control, $n = 5$, $P < 0.05$) and F_R (on average down to 78.3 ± 5.5% as compared to the control, $n = 5$, $P < 0.05$) (Fig. 10, *A*). Contrary to the C-1191 compound, thiacalix[4]arene chalcone amide C-1192 affected the time parameters (τ_0, τ_C and τ_R) of the spontaneous contractions considerably and evenly (Fig. 10, *B*), which may demonstrate the activation of the processes of the uptake and pumping out of Ca^{2+} ions from the cytoplasm of myocytes.

Also, C-1192 (contrary to C-1191) did not affect the absolute values of the maximal velocities of the contraction and relaxation phases (Fig. 10, *C*), but setting their norms with the consideration of the

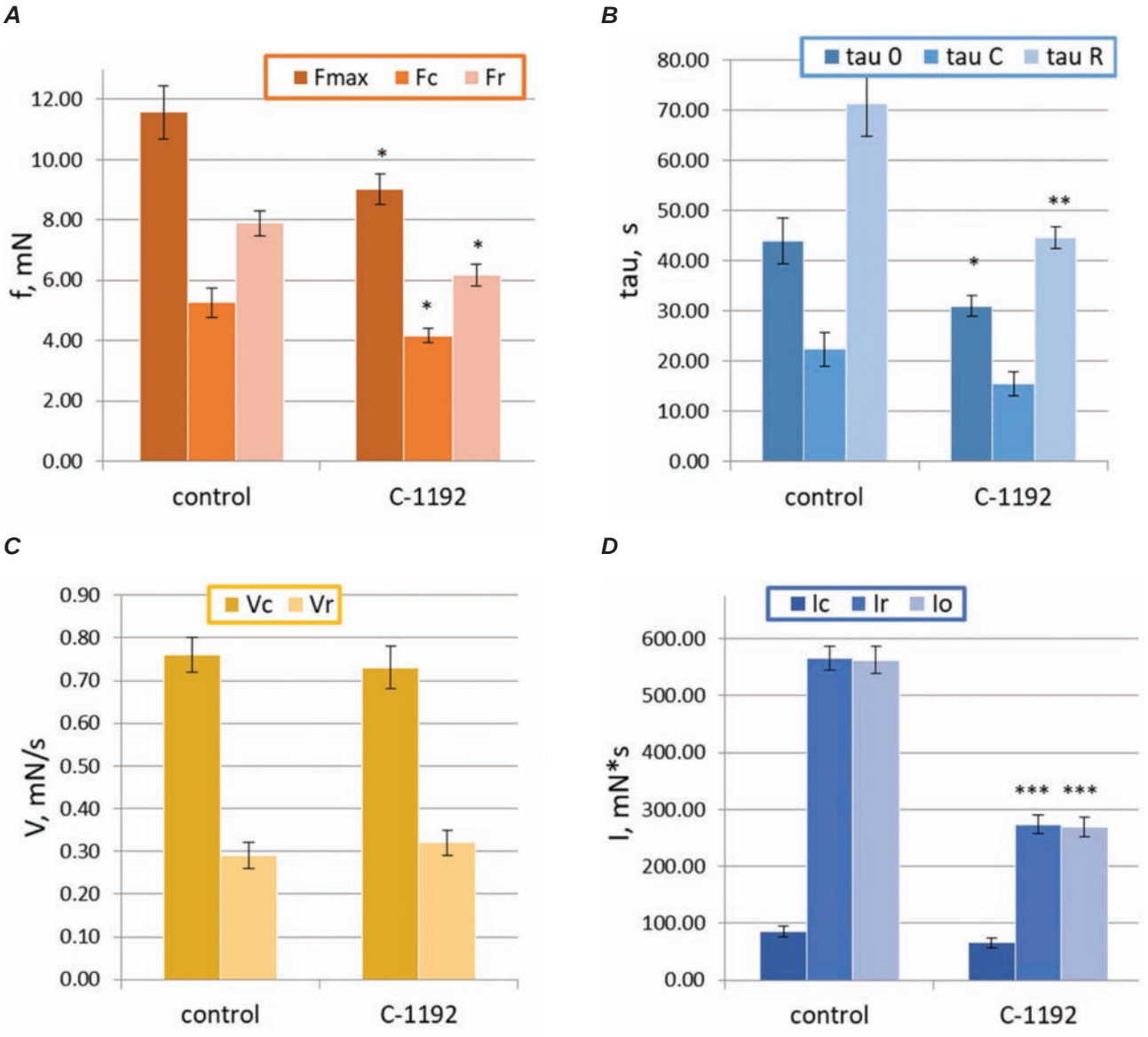


*Fig. 10. The mechanokinetic parameters of the spontaneous contractile activity of rat myometrium in the control and under the previous incubation on the background of calix[4]arene C-1192 (10 μM): **A** – force parameters (F_{max}, F_C and F_R); **B** – time parameters (τ_0, τ_C and τ_R); **C** – velocity parameters (V_C and V_R); **D** – impulse parameters (I_0, I_C and I_R). n = 5; *P < 0.05, **P < 0.01, ***P < 0.001 – significant against control*

amplitude was accompanied by their significant increase (the normalized maximal velocity of the contraction phase – on average up to 137.2 ± 4.1% and the normalized maximal velocity of the relaxation phase – on average up to 152.9 ± 8.6%, both $n = 5$, $P < 0.05$), which may be related to the intensification of the processes of the uptake and pumping out of Ca^{2+} ions from the cytoplasm of myocytes. All the impulse parameters of spontaneous contractions (I_0, I_C and I_R) decreased considerably on the background of C-1192 (Fig. 10, *D*).

Therefore, contrary to C-1191, thiacalix[4]arene chalcone amide C-1192 modified the uterine cycle considerably and increased the total efficiency of the spontaneous contractile activity in the myometrium. Also, the C-1192 compound (but not C-1191) caused a decrease in all force (F_{max}, F_C and F_R) and some time (τ_0 and τ_R) and impulse (I_0 and I_R) parameters. So, the effects of C-1191 cannot be considered specific in terms of some systems involved in the realization of the cellular mechanisms of the contraction-relaxation cycle.

Since thiacalix[4]arene chalcone amides C-1191 and C-1192 induced a considerable modification of the contractile activity in the myometrium, we analyzed the mechanokinetic effects of calix[4]arene chalcone amide C-1011 (10 μM). Similar to other sulfur-containing analogs, the C-1011 compound induced an increase in the basal tension of the myometrium preparations. On the background of calix[4]arene chalcone amide C-1011, there was also the activation in the myometrium motility, demonstrated in the rise in the amplitude and frequency of spontaneous contractions. The C-1011 compound did not cause disruptions in the uterine cycle but induced a considerable decrease in the duration of pauses between contractions. The changes in all the mechanokinetic parameters (force, time, velocity, and impulse) of the spontaneous contractions in myometrium under the effect of calix[4]arene chalcone amide C-1011 were similar to those induced by C-1191, in terms of the direction of changes but the former were manifested much more vividly (Fig. 11).

The mechanism of the activation of spontaneous contractions in uterine myocytes has not yet been completely studied; it is assumed that either Kit-negative pacemaker cells or myocytes themselves are responsible for the pacemaker activity [17]. It is known that the pacemaker mechanisms involve the processes of the uptake of the extracellular Ca^{2+} ions via potential-governed channels of L- and T-types (the association with the frequency but not amplitude of contractions was proven for T-channels) [18-20], Ca^{2+}-activated Cl^--channels [17], and

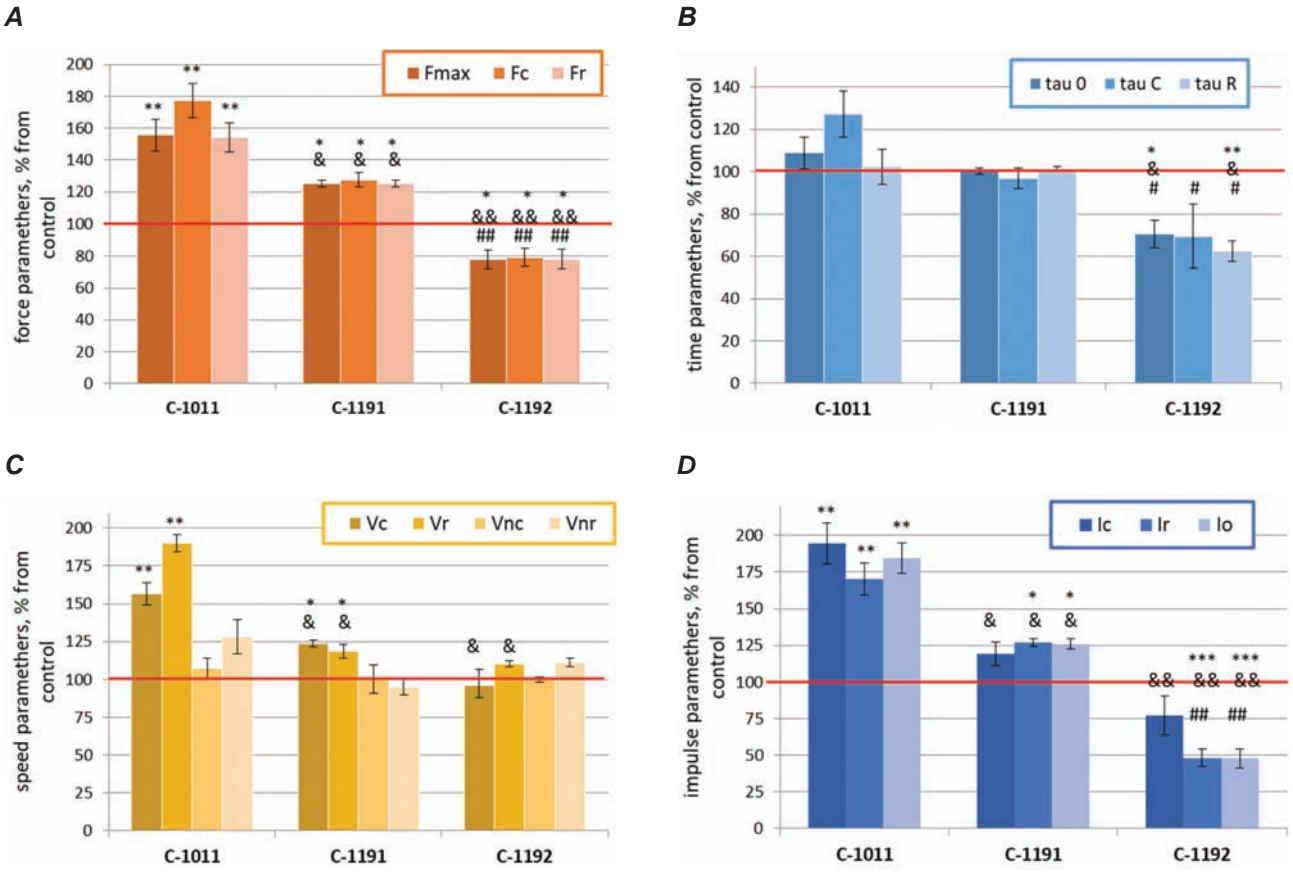


*Fig. 11. The summary of relative (against the relevant control indices) values of the mechanokinetic parameters of the spontaneous contractile activity in rat myometrium under the effect of calix[4]arene chalcone amide C-1011 and thiacalix[4]arene chalcone amides C-1191 and C-1192 (all the compounds were 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, and the maximal velocities, normalized with the consideration of the amplitude of contractions – V_{nC} and V_{nR}); **D** – impulse parameters (I_0, I_C and I_R). n = 5; *P < 0.05, **P < 0.01 – significant against control; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ – significant against C-1011; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ – significant against C-1191*

the release of Ca^{2+} ions by the intracellular depots, including mitochondria, the activity of which impacts both the amplitude and frequency of spontaneous contractions [21, 22]. Therefore, we can assume that generally the modulation of the contractile activity in myometrium by thiacalix[4]arene chalcone amides C-1191 and C-1192 is related to their ability to affect the functioning of the mitochondria. The differences in the mechanokinetic effects of these compounds may be conditioned by the ability of the latter, being in the concentration of 10 μM, to have different values effects on the functional activity of mitochondria.

Therefore, it was found that:

Thiacalix[4]arenes C-1191 and C-1192 did not affect the ionized Ca concentration in the myometrium mitochondria under short-term incubation.

Thiacalix[4]arenes C-1191 and C-1192 did not affect the hydrodynamic diameter of rat myometrium mitochondria, except for calix[4]arene C-1192, which enlarged the size of mitochondria in the concentration of 10 μM.

The incubation of the digitonin-perforated myometrium cells with 1 μM C-1191 or 1 μM C-1192 increased the fluorescence intensity of the potential-sensitive TMRM probe as compared to the control, which may demonstrate the hyperpolarizing effect of these compounds on mitochondria.

Thiacalix[4]arenes C-1191 and C-1192, similar to compound C-1011, induced the increase in the basal tension of smooth muscle preparations of rat myometrium.

Thiacalix[4]arene C-1191 did not change the uterine cycle and the total efficiency of the myometrium functioning, but, similar to compound C-1011, reliably and evenly increased all force (F_{max}, F_C and F_R) and velocity (V_C and V_R) parameters as well as some impulse parameters (I_0 and I_R).

Contrary to C-1191, thiacalix[4]arene C-1192 modified the uterine cycle considerably and increased the total efficiency of the spontaneous contractile activity in the myometrium. Also, the C-1192 compound (but not C-1191) caused a decrease in all force (F_{max}, F_C and F_R) and some time (τ_0 and τ_R) and impulse (I_0 and I_R) parameters.

The mechanokinetic effects of thiacalix[4]arenes C-1191 and C-1192 cannot be considered specific regarding some systems involved in the realization of the contraction-relaxation cycle; it can be predicted that the changes in some mechanokinetic parameters of the rhythm activity of the myometrium were conditioned by the change in the involvement of mitochondria in the regulation of the contractile function of myometrium.

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

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ВПЛИВ ТІАКАЛІКС[4]АРЕН ХАЛКОН АМІДІВ НА СКОРОЧЕННЯ МІОМЕТРІЯ

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Каліксарени є макроциклічними сполуками, біохімічні ефекти яких активно вивчаються. У даній роботі синтезовано тіакалікс[4]арен халкон аміди С-1191 і С-1192, які мають у своїй структурі атом сірки та різне просторове розташування халкон амідних груп, і вивчено їх вплив на функціонування міометрія. Експерименти проводили з використанням препаратів гладкої мускулатури матки щурів, ізольованих мітохондрій міометрія та пермеабілізованих клітин міометрія. Відносне значення мембранного потенціалу мітохондрій ($\Delta\psi$) аналізували за допомогою вольтаж-чутливого флуоресцентного зонда TMRM. Спонтанну скоротливу активність вивчали тензометричним методом із наступним механокінетичним аналізом. Показано, що С-1191 і С-1192 індукують гіперполяризацію мітохондрій і підвищують базальну напругу гладком'язового препарату міометрія. Тіакаліксарен[4]арен С-1191 не змінював матковий цикл, але підвищував силові, швидкісні та імпульсні параметри скоротливої діяльності м'язів. У той же час, С-1192 суттєво

модифікував матковий цикл, підвищував загальну ефективність спонтанної скорочувальної активності міометрія та знижував як силові, так і часові та імпульсні параметри. Зроблено висновок, що зміни механокінетичних параметрів скорочувальної активності міометрія, індуковані С-1191 та С-1192, визначаються функціональною активністю мітохондрій.

Ключові слова: тіакалікс[4]-арен халкон аміди, мітохондрії, мембранний потенціал мітохондрій, скоротлива активність міометрія, механокінетичний аналіз.

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