EXPERIMENTAL WORKS

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Ca^{2+}-DEPENDENT MECHANISMS OF MITOCHONDRIA-TARGETED CALIX[4]ARENE C-772 EFFECT ON UTERINE SMOOTH MUSCLE CONTRACTILE ACTIVITY

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Background. *Identification of non-toxic exogenous compounds capable of selectively influencing intracellular Ca^{2+}-dependent processes and smooth muscle contractility remains an important task of molecular biotechnology. Calix[4]arenes are considered promising modulators of cellular functions, and calix[4]arene C-772 is suggested to selectively effect mitochondrial Ca^{2+} handling.* ***Objective.*** *The aim of this study was to evaluate the effect of calix[4]arene C-772 on Ca^{2+} transport in mitochondria of uterine smooth muscle, nitric oxide production in myocytes, and the contractile activity of myometrial strips.* ***Methods.*** *Non-pregnant Wistar rats were used in experiments, confocal microscopy, spectrofluorimetry, flow cytometry, and mechanokinetic analysis were applied.* ***Results.*** *Mitochondrial localization of calix[4]arenes in myocytes was confirmed by colocalization of the calix[4]arene–FITC conjugate C-1308 with Mitotracker Orange CM-H_2TMRos. Calix[4]arene C-772 (10 μM) interacted with cardiolipin in mitochondrial membranes, inhibited nitric oxide synthesis, reduced Ca^{2+} accumulation in isolated mitochondria and increased the amplitude of rat myometrial smooth muscle spontaneous contractions. Mechanokinetic analysis demonstrated increased force, velocity, and impulse parameters of contraction–relaxation cycles.* ***Conclusions.*** *These findings indicate that calix[4]arene C-772 at micromolar concentrations can serve as an effective modulator of mitochondrial functional activity and uterine smooth muscle contractility.*

K e y w o r d s: mitochondria, calix[4]arene, smooth muscle, myometrium, calcium, cardiolipin, nitric oxide.

Disorders of smooth muscle contractility and the pathological conditions associated with them represent a significant medical and social problem in developed countries. In particular, uterine atony or hypertonicity may lead to weak labor activity or, conversely, to preterm labor and miscarriages. The established relationship between smooth muscle contractile dysfunction and impaired mitochondrial activity has led to the concept of mitochondrial diseases as an important cause of disorders of smooth muscle organs [1-3].

Mitochondria play a key role in Ca^{2+} signaling and Ca^{2+}-dependent contraction of smooth muscles [4-6]. They function as an efficient intracellular Ca^{2+} store due to their ability to accumulate and release substantial amounts of this cation. In our previous studies, the addition of the protonophore CCCP to isolated uterine myocytes, which dissipates the electrochemical proton gradient across the inner mitochondrial membrane and thereby inhibits energy-dependent Ca^{2+} accumulation by mitochondria, resulted in a significant increase in Ca^{2+} concentra-

tion in the mitochondrial matrix [7]. Disruption of the coordinated functioning of Ca^{2+} transport systems located in the inner mitochondrial membrane may lead to Ca^{2+} overload of the matrix and mitochondrial dysfunction [1-3, 8-10]. Given the crucial role of Ca^{2+} in mitochondrial function and smooth muscle physiology, the search for non-toxic exogenous compounds capable of selectively modulating mitochondrial Ca^{2+} transport systems has become an important task in molecular biotechnology.

In recent years, considerable attention has been paid to macrocyclic nanosized compounds, particularly calix[4]arenes, whose macrocyclic cavity exceeds 1 nm^3 [11]. Available data suggest that, at relatively low (micromolar) concentrations, certain groups of calix[4]arenes can influence the functional activity of myometrial mitochondria [12, 13]. Their advantages as tools for biochemical research include low toxicity and relatively inexpensive synthesis.

The directed synthesis and screening of compounds intended as modulators of mitochondrial activity, mitoprotectors, or experimental tools are often based on specific biophysicochemical properties of mitochondria. An important criterion for identifying potential mitochondria-targeting compounds is their interaction with cardiolipin, a phospholipid enriched in the inner mitochondrial membrane [14]. In addition, efficient cellular penetration and interaction with mitochondria require a small number, or the absence, of negative charges, which otherwise hinder accumulation across membranes with a high negative electric potential. Both factors should be considered when searching for calix[4]arenes suitable for biochemical studies of mitochondria and targeted modulation of their activity.

Previous studies have shown that calix[4]arene C-772 (Fig. 1) in micromolar concentrations exerts only a weak effect on Ca^{2+}-transport systems localized in the plasma membrane and sarco(endo)plasmic reticulum of myometrial cells. Moreover, it does not affect the ATP-hydrolase activity of myometrial myosin head (subfragment S1) within this concentration range [15, 16]. These observations suggest that the modulation of myometrial functional activity by this compound may be associated with its influence on mitochondrial Ca^{2+} transport and Ca^{2+}-dependent processes. One such process is NO (nitric oxide) synthesis in mitochondria, which represents an important source of this regulatory molecule in uterine myocytes [17, 18]. NO is known to induce relaxation of smooth muscles, including the uterus of humans and animals, during various functional states [18, 19].

Therefore, the aim of the present study was to investigate the effect of calix[4]arene C-772 on Ca^{2+} transport in isolated mitochondria of uterine smooth muscle, NO production in myocytes, and the contractile activity of myometrial strips, as well as to perform a multiparametric analysis of contraction–relaxation cycles, including evaluation of their force, temporal, impulse, and velocity parameters.

Materials and Methods

Chemical synthesis. Calix[4]arene C-772 (Fig. 1, $C_{58}H_{62}F_6N_4O_8S_2$, 5,11-di(trifluoromethyl-(phenylsulfonylimino)methylamino)-17,23-di-*tert*-butyl-25,26-dipropoxy-27,28-dihydroxycalix[4]arene) was synthesized by the dropwise addition of a solution of the diaminocalix[4]arene precursor (1.0 mmol) and triethylamine (2.0 mmol) in dry benzene (10 ml) to a stirred solution of N-sulfonylimidoyl chloride (2.1 mmol) in dry benzene (15 ml) over 15 min. The reaction mixture was stirred for 16 hours at room temperature. The precipitate of the target product together with triethylammonium chloride was filtered off and washed successively with benzene (10 ml) and methanol–water (2:1, v/v, 15 ml). The solid was air-dried for 3 h and further dried in vacuo for 4 h. The product was obtained as a colorless crystalline solid in 84% yield; m.p. = 265–267°C. ^{1}H NMR ($(CD_3)_2SO$, 299.94 MHz), δ (ppm): 1.10 (t, J = 7.5 Hz, 6H, O–CH_2–CH_2–$C\underline{H}_3$), 1.12 (s, 18H, tert-Bu), 2.01 (m, 4H, O–CH_2–$C\underline{H}_2$–CH_3), 2.94 (d, J = 11.0 Hz, 1H, ArCHeqAr), 3.32 (br s, 2H, $ArCH_{eq}Ar$), 3.54 (d, J = 12.5 Hz, 1H, $ArCH_{eq}Ar$), 3.82 (m, 2H, O–CH_2–CH_2–CH_3), 4.11 (br s, 4H, O–CH_2–CH_2–CH_3, $ArCH_{ax}Ar$), 4.30 (d, J = 12.0 Hz, 2H, $ArCH_{ax}Ar$), 6.91 (br s, 4H, C_6H_2–OPr), 7.15 (br s, 2H, C_6H_2–OH), 7.40 (s, 2H, C_6H_2–OH), 7.57 (m, 6H, SO_2–Ph), 7.85 (s, 4H, SO_2–Ph), 8.60 (br s, 2H, OH), 11.01 (br s, 2H, NH). ^{19}F NMR ($CDCl_3$, 188.13 MHz), δ (ppm): –66.27 (s, CF3). ^{13}C NMR ($CDCl_3$, 150.83 MHz), δ (ppm): 11.20, 21.48, 23.46, 30.85, 78.48, 78.50, 120.46 (q, J = 19.07 Hz, N–C=N), 125.63 (q, J = 65.64 Hz, CF3), 127.78, 128.25, 128.31, 129.27, 129.74, 129.91, 133.97, 137.84, 144.09, 149.33, 149.78, 152.14. Elemental analysis: calcd for $C_{58}H_{62}F_6N_4O_8S_2$ (%): C, 62.13; H, 5.57; N, 5.00; S, 5.72; found (%): C, 62.01; H, 5.40; N, 4.80; S, 5.92. HRMS (ESI$^-$-QTOF): Mass calcd for $C_{58}H_{62}F_6N_4O_8S_2$: 1120.3913, found: 1120.3885; $[M-H]^-$ calcd m/z: 1119.3840, found m/z: 1119.3820; $[M-2H]^{-2}$ calcd m/z: 559.1884, found m/z: 559.1876.

Calix[4]arene–FITC conjugate C-1308 (Fig. 1, $C_{84}H_{76}N_4O_{14}S_2$, 5,17-di(*N*-(3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-yl)-thioureido)-11,23-di-*tert*-butyl-25,27-dipropoxy-26,28-dihidroxycalix[4]arene) was synthesized by adding fluorescein isothiocyanate (FITC, 100 mg, 0.2568 mmol) dropwise to a solution of diaminocalix[4]arene bis(trifluoroacetate) (115 mg, 0.128 mmol) and diisopropylethylamine (40 mg, 0.309 mmol) in anhydrous tetrahydrofuran (5 ml) at room temperature. The reaction mixture was stirred for 14 h, after which it was filtered and the solvent was removed under reduced pressure. The resulting residue was dissolved in a chloroform–water mixture; the organic phase was washed with water and brine, dried over Na2SO4, and evaporated *in vacuo*. The crude product was purified by column chromatography (dichloromethane–methanol, 100:5, v/v; R_f = 0.34) to yield a yellow crystalline solid (40 mg, 21.6%; m.p. > 300°C). ^{1}H NMR (CD_3OD, 301 MHz), δ (ppm): 1.02 (broadened s, 18H, tert-Bu), 1.29 (broadened t, 6H, O-CH_2-CH_2-CH_3), 2.11 (m, 4H, O-CH_2-CH_2-CH_3), 3.49 (d, J = 12.2 Hz, 4H, $ArCH_{eq}Ar$), 3.99 (m, 4H, O-CH_2-CH_2-CH_3), 4.32 (d, J = 12.2 Hz, 4H, $ArCH_{ax}Ar$), 6.54-6.72 (m, 14H, xanth+benzofur), 7.15+7.19 (two s, 4H+4H, ArH-calix), 7.89 (s, 4H, benzofur), 7.96 (s, 2H, OH). HRMS (ESI$^+$-QTOF): Mass calcd for $C_{84}H_{76}N_4O_{14}S_2$: 1428.4799, found: 1428.4791; $[M+H]^+$ calcd m/z: 1429.4872, found m/z: 1429.4864.

The NMR spectra were recorded on a Varian VXR-400 spectrometer; operating frequencies are specified in the corresponding spectral descriptions. Chemical shifts (δ) are reported in parts per million (ppm) relative to the solvent signal or tetramethylsilane (TMS), and spin–spin coupling constants (J) are given in hertz (Hz). Thin-layer chromatography (TLC) was performed on silica gel plates (Macherey-Nagel, AluGram Xtra, G/UV_{254}), and column chromatography was carried out using silica gel (S60, ROCC, Belgium; particle size 0.4–0.6 mm). Melting points were determined using a Boetius apparatus. High-resolution mass spectrometry (HRMS) analysis was performed on an Agilent 1200 Series – Agilent G6545A Q-TOF LC/MS system (USA) equipped with a Zorbax SB-C8 column.

Compounds C-772 and C-1308 were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions of varying concentrations and stored in the frozen state. For experiments, appropriate aliquots were added to the incubation medium immediately before measurements. The final DMSO concentration did not exceed 0.5% (v/v); control samples received an equivalent volume of the solvent.

Animals. Experiments were performed on female non-pregnant Wistar rats weighing 150–180 g maintained under standard vivarium conditions at constant temperature with free access to food and water. All procedures complied with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986) [20] and the Law of Ukraine "On the Protection of Animals from Cruelty", and were approved by the Bioethics Commission of the

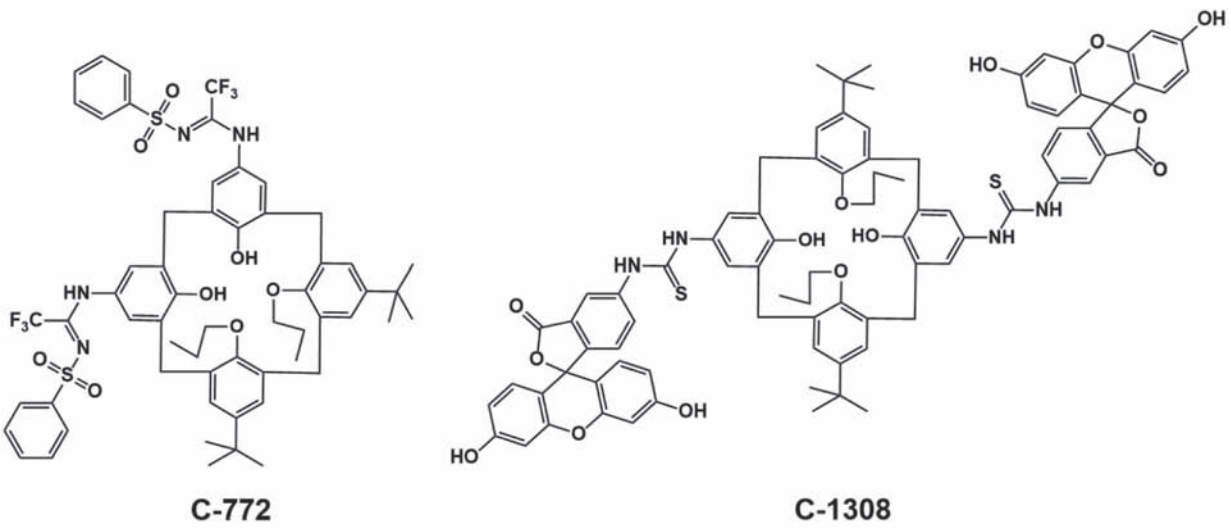

Fig. 1. Chemical structures of calix[4]arene C-772 and calix[4]arene–FITC conjugate C-1308

Palladin Institute of Biochemistry of the NAS of Ukraine (Order No. 15, September 5, 2025). Rats were anesthetized with chloroform inhalation and subsequently decapitated.

Isolation of uterine myocytes. Uterine myocytes were isolated by the Mollard method using collagenase (type IA) and soybean trypsin inhibitor [21]. Experiments were carried out in Hanks' physiological solution of the following composition (mM): 136.9 NaCl, 5.36 KCl, 0.44 KH_2PO_4, 0.26 $NaHCO_3$, 0.26 Na_2HPO_4, 0.03 $CaCl_2$, 5.5 glucose, and 10 HEPES (pH 7.4 at 37 °C).

Isolation of myometrial mitochondria. Mitochondria were isolated from rat myometrium by differential centrifugation as described previously [7]. The mitochondrial pellet was resuspended in a medium of the following composition (mM): 250 sucrose and 10 HEPES (pH 7.3 at 4°C), supplemented with 0.1% bovine serum albumin (BSA). Protein concentration in the mitochondrial fraction was determined by the Bradford method [22].

Confocal microscopy. Confocal imaging, cell immobilization, washing of non-adherent myocytes, and other experimental procedures were performed in Hanks' solution. Fluorescence images were acquired with an LSM 510 META confocal laser scanning microscope (Carl Zeiss, Germany). Myocytes were immobilized on poly-L-lysine–coated surfaces. Fluorescence of MitoTracker Orange CM-H2TMRos (200 nM) was excited at 543 nm and recorded using a BP 560–615 emission filter. Calix[4]arene–FITC conjugate C-1308 (10 μM) was excited at 488 nm with emission detected through a BP 505–530 filter. Hoechst 33342 (50 nM) fluorescence was excited at 405 nm and detected with a BP 420–480 filter. Fluorescent probes were loaded for 15 min at 24 °C. Image acquisition was performed in MultiTrack mode.

Study of changes in NAO fluorescence. Changes in acridine orange 10-nonyl bromide (NAO) fluorescence were measured using a QuantaMaster 40 PTI spectrofluorimeter (Canada) operated with FelixGX 4.1.0.3096 software. Measurements were performed in an incubation medium of the following composition (mM): 20 HEPES (pH 7.4 at 37°C), 2 K^+-phosphate buffer, 125 KCl, 25 NaCl, 5 sodium succinate, 5 sodium pyruvate, supplemented with 100 nM NAO. Fluorescence was recorded at λ_{ex} = 488 nm and λ_{em} = 530 nm or 510–550 nm.

Measurement of NO synthesis in myocytes. NO production in isolated myocytes was analyzed by flow cytometry using a DxFLEX flow cytometer (Beckman Coulter, USA) equipped with an argon laser (λ_{ex} = 488 nm) and CytExpert software. Fluorescence was detected at 525 nm. All experimental procedures, including loading myocytes with 5 μM DAF-FM, were carried out in Hanks' solution.

Measurement of NO synthesis in mitochondria. NO production in mitochondria was measured using the fluorescent probe DAF-FM (λ_{ex} = 488 nm, λ_{em} = 525 nm) with a QuantaMaster 40 PTI spectrofluorimeter. Mitochondria were loaded with 5 μM DAF-FM-DA for 30 min at 25 °C in a medium of the following composition (mM): 10 HEPES (pH 7.4 at 25°C) and 250 sucrose, supplemented with 0.1% BSA and 0.02% Pluronic F-127. Measurements were performed in an incubation medium of the following composition (mM): 20 HEPES (pH 7.4 at 37°C), 2 K^+-phosphate buffer (pH 7.4 at 37°C), 125 KCl, 25 NaCl, 5 sodium succinate, 5 sodium pyruvate, 0.01 NADPH, 0.01 tetrahydrobiopterin, 0.05 L-arginine, and 0.1 Ca^{2+}. The mitochondrial fraction contained 15–20 μg protein per sample. Incubation time was 15 min.

Measurement of mitochondrial ionized Ca^{2+} content. Mitochondria were loaded with the Ca^{2+}-sensitive fluorescent probe Fluo-4 AM (2 μM; λ_{ex} = 490 nm, λ_{em} = 525 nm) for 30 min at 37°C in a medium of the following composition (mM): 10 HEPES (pH 7.4 at 37°C) and 250 sucrose, supplemented with 0.1% BSA and 0.02% Pluronic F-127. Fluorescence was recorded using a QuantaMaster 40 PTI spectrofluorimeter. Ca2+ accumulation was measured in an incubation medium of the following composition (mM): 20 HEPES (pH 7.4 at 37°C), 250 sucrose, 2 K^+-phosphate buffer, 3 $MgCl_2$, 3 ATP, and 5 sodium succinate. The Ca^{2+} concentration was 0.08 mM.

Tensometric experiments. Tensometric experiments were performed on longitudinal smooth muscle strips isolated from uterine horns. Muscle preparations (≈2×10 mm) were placed in a 2 ml chamber perfused with Krebs solution at a flow rate of 5 ml/min and maintained at 37°C. Preparations were passively stretched to 10 mN and allowed to equilibrate for 1 h until stable spontaneous contraction amplitude and frequency were achieved. Contractile activity was recorded in isometric mode using a force transducer, and signals were digitized via an analog-to-digital converter. The Krebs solution had the following composition (mM): 120.4 NaCl, 5.9 KCl, 15.5 $NaHCO_3$, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, and 11.5 glucose (pH 7.4). Appropriate aliquots of com-

pound C-772 were added to the incubation medium immediately before measurements, with the final DMSO concentration not exceeding 0.1% (v/v), while control samples received an equivalent volume of the solvent.

Mechanokinetic analysis of contractions. Spontaneous contractile activity of smooth muscle preparations was analyzed using a previously developed empirical multiparameter method for mechanokinetic analysis [23]. The full profile of individual spontaneous isometric contractions was linearized in the coordinates [$\ln(f_R/f_C)$; $\ln(1+\Delta t/t)$], where f and t denote instantaneous force and time within a contraction cycle (C – contraction phase, R – relaxation phase). F_C and F_R correspond to force values at the inflection points of the mechanogram during contraction (from force onset to the maximal value, F_{max}) and relaxation (from F_{max} at τ_0 to the basal level), respectively, whereas Δt represents a fixed time interval (15–50 s). Linearization plots yielded constants *k* and *n*, which were used to calculate the parameters of contraction–relaxation cycles: time (τ_0, τ_C, τ_R), force (F_{max}, F_C, F_R), velocity (V_C, V_R), and impulse (I_0, I_C, I_R). V_C and V_R correspond to maximal velocities of contraction and relaxation, whereas I_{max}, I_C, and I_R represent force impulses at the amplitude and maximal velocities of contraction and relaxation. To estimate the overall efficiency of smooth muscle contractile activity, contractile indices expressed in Montevideo units (MU) and Alexandria units (AU) were calculated. MU were defined as the product of the mean contraction amplitude and frequency over 10 min, whereas AU represented the product of MU and the mean contraction duration over the same time interval.

Estimation of myocyte hydrodynamic diameter. The hydrodynamic diameter distribution (characteristic size) of myocytes was determined by photon correlation spectroscopy [24] using a Zeta-Sizer-3 analyzer (Malvern Instruments, Great Britain). The instrument was equipped with a Multi8 computing correlator (type 7032 ce) and a helium–neon LGH-111 laser (λ = 633 nm, 25 mW). Scattered light from cell suspensions was recorded for 10 min at 1-min intervals at 24°C and a scattering angle of 90°. Autocorrelation functions were processed using PCS-Size software (mode v1.61). All measurements were carried out in Hanks' solution.

Statistical analysis. Statistical analysis was performed using MS Excel and Origin 2021 software. Normality of data distribution was tested using the Shapiro–Wilk test. Differences between mean values were evaluated using paired Student's t-test and one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The reliability of the data approximation by a linear function was checked using Fisher's F-test; in all cases, the determination coefficients (R^2) were at least 0.97 in all cases. Differences were considered statistically significant at $P < 0.05$. Data are presented as mean ± S.E.M.

Chemicals and reagents. The following reagents were used: HEPES, glucose, sucrose, sodium succinate, sodium pyruvate, BSA, poly-L-lysine, collagenase type IA, ATP, Pluronic F-127, $CaCl_2$, DAF-FM-DA, NADPH, and tetrahydrobiopterin (Sigma, USA); L-arginine, FITC, and NAO (Sigma-Aldrich, USA); soybean trypsin inhibitor (Fluka, Switzerland); MitoTracker Orange CM-H2TMRos (Invitrogen, USA); and Fluo-4 AM (Molecular Probes, USA). All other reagents were of analytical grade and produced in Ukraine. Solutions were prepared with bidistilled water with a specific electrical conductivity not exceeding 2.0 µS/cm, measured using an OK-102/1 conductometer (Hungary).

Results and Discussion

For calix[4]arene C-772 to interact with mitochondria, it must first penetrate the plasma membrane and distribute within the cytoplasm of myocytes. This process requires sufficient hydrophobicity of the compound, which is ensured by the presence of phenylsulfanilamide substituents and the absence of significant negative charges in the molecule (Fig. 1).

In this context, the structural features of calix[4]arene C-772, including multiple aromatic rings, suggest its potential intrinsic fluorescence. Indeed, the compound exhibits a characteristic fluorescence maximum at approximately 400 nm, which is distinct from the autofluorescence of adenine nucleotides and other endogenous cellular components. This property enables monitoring of C-772 interaction with subcellular structures (Fig. 2, *A*). In the studied concentration range, no self-quenching of the fluorescence signal was observed in the working solution (Fig. 2, *B*).

Upon addition to myocytes, calix[4]arene C-772 interacts with the plasma membrane, likely involving the hydrophobic lipid bilayer, which is accompanied by an increase in fluorescence intensity. As the compound subsequently enters the cytosol, a gradual decrease in fluorescence is observed (Fig. 3),

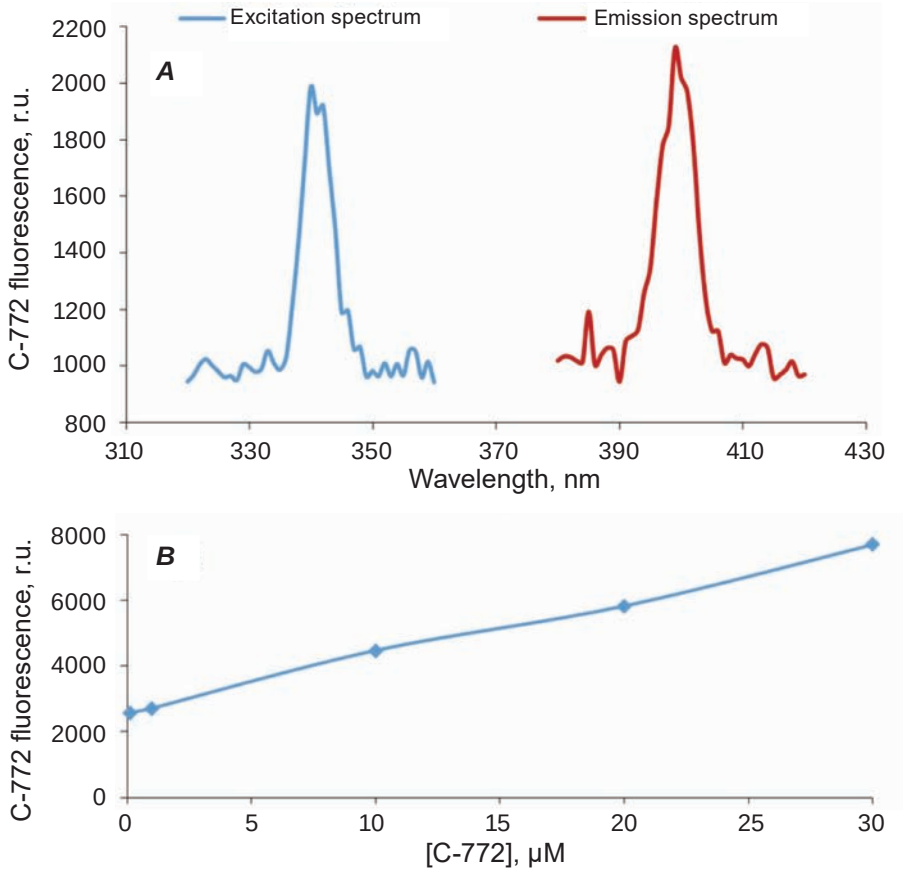

Fig. 2. Excitation and fluorescence spectra of calix[4]arene C-772 (A) and concentration dependence of its fluorescence intensity in the working medium (B)

indicating changes in its microenvironment and/or intracellular distribution.

Interaction of the fluorescent probe NAO (acridine orange 10-nonyl bromide) with isolated mitochondria of myometrium results in an increase in its fluorescence intensity due to binding to cardiolipin enriched in the inner mitochondrial membrane (Fig. 4, *A)*, consistent with its use as a mitochondria-specific probe [25]. In the presence of calix[4]arene C-772, a decrease in NAO fluorescence is observed (Fig. 4, *B*). This suggests that C-772 interacts with cardiolipin, thereby reducing NAO binding and leading to attenuation of its fluorescence signal in mitochondria. This effect may reflect competition between C-772 and NAO for cardiolipin binding sites. Alternatively, C-772 may alter the physicochemical properties of the inner mitochondrial membrane, thereby affecting NAO fluorescence. Such interactions may, in turn, influence mitochondrial Ca^{2+} handling and associated signaling processes.

The ability of calix[4]arene C-772 to penetrate myocytes and interact with mitochondria is supported by confocal microscopy data. Colocalization of the mitochondrial probe MitoTracker Orange CM-H2TMRos with calix[4]arene–FITC conjugate C-1308 (Fig. 1) was observed in myocytes (Fig. 5), indicating mitochondrial localization of calix[4]arene derivatives.

Covalent labeling with fluorescein isothiocyanate (FITC), which exhibits strong fluorescence in the green spectral region, is widely used to study intracellular localization of bioactive compounds. The C-1308 derivative used in this study displayed high fluorescence intensity (Fig. 5), while the presence of a stable thioamide linkage prevents enzymatic cleavae and release of FITC within the cell.

Taken together, these results indicate that calix[4]arenes can localize in mitochondria of myocytes. This is consistent with previous observations demonstrating the ability of related compounds, including C-956, to penetrate myocytes [12].

Previous studies have shown that myometrial mitochondria possess intrinsic NO synthase activity and represent a major source of nitric oxide in uteri-

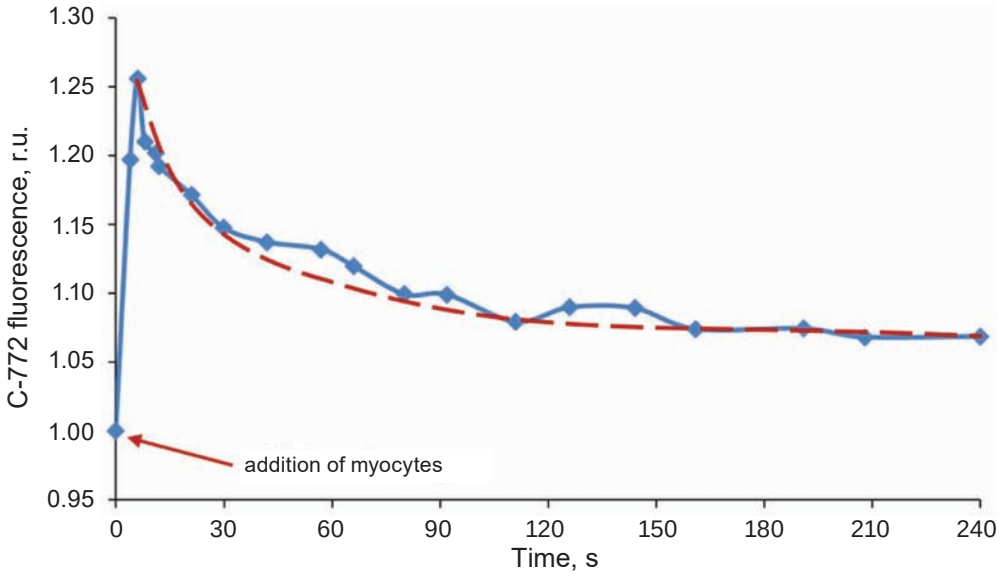

Fig. 3. Time-dependent changes in fluorescence of calix[4]arene C-772 (30 μM) during interaction with myocytes. Representative data from a typical experiment (n = 3)

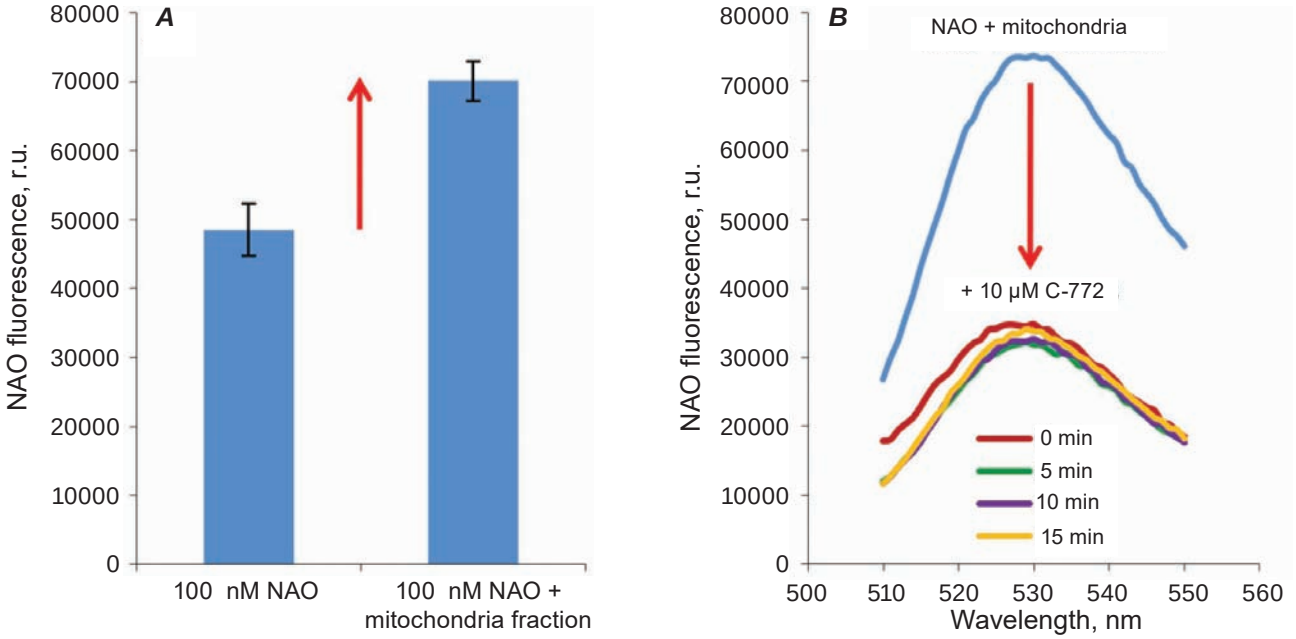

*Fig. 4. Changes in NAO fluorescence upon interaction with mitochondria (**A**) and in the presence of 10 μM calix[4]arene C-772 (**B**); n = 5 (**A**); representative traces (**B**)*

ne myocytes [17]. In the present study, calix[4]arene C-772 (10 and 30 μM) was found to inhibit NO production in myocytes, with only minor dependence on the concentration of exogenous Ca^{2+} (Fig. 6, *A*). The magnitude of this effect did not differ significantly between the tested concentrations of C-772.

A similar decrease in NO synthesis was observed in isolated mitochondria (Fig. 6, *B*), supporting the contribution of mitochondria to NO production in these cells and indicating that C-772 acts, at least in part, at the mitochondrial level. Given that mitochondrial NO production is closely associa-

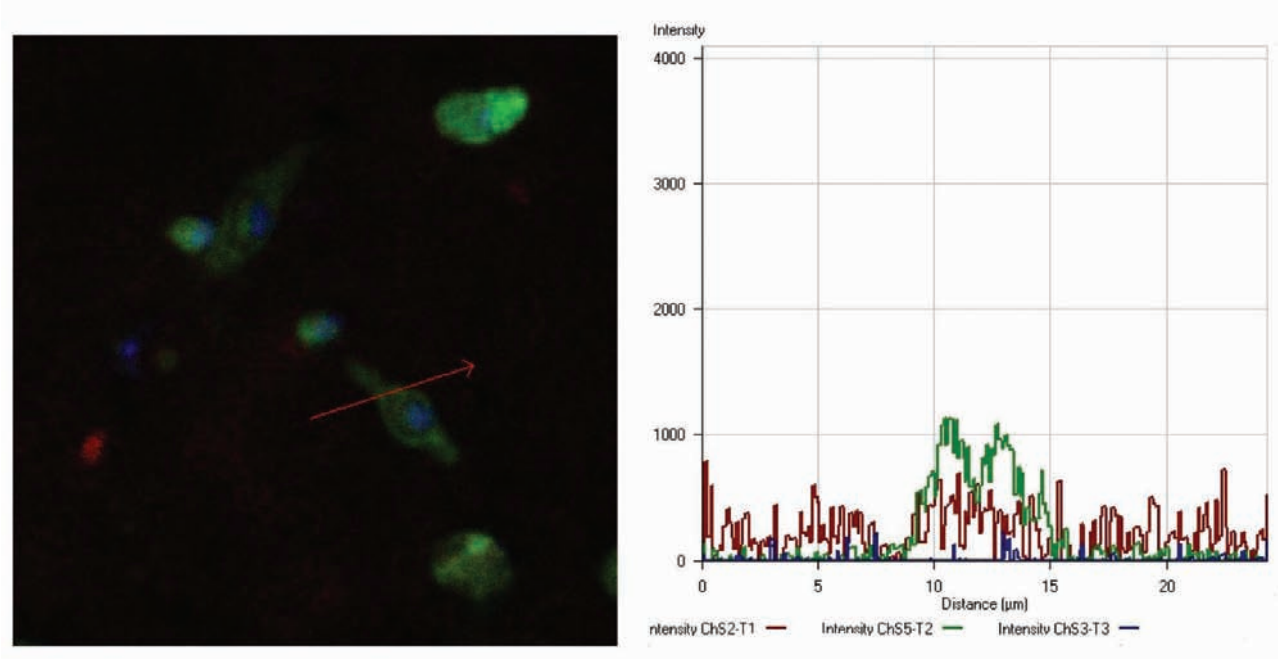

Fig. 5. Colocalization of calix[4]arene–FITC conjugate C-1308 (10 µM, green) and MitoTracker Orange CM-H2TMRos (200 nM, red) in a myocyte. Distribution profiles of fluorescence signals are shown. Nuclei are stained with Hoechst 33342 (50 nM, blue)

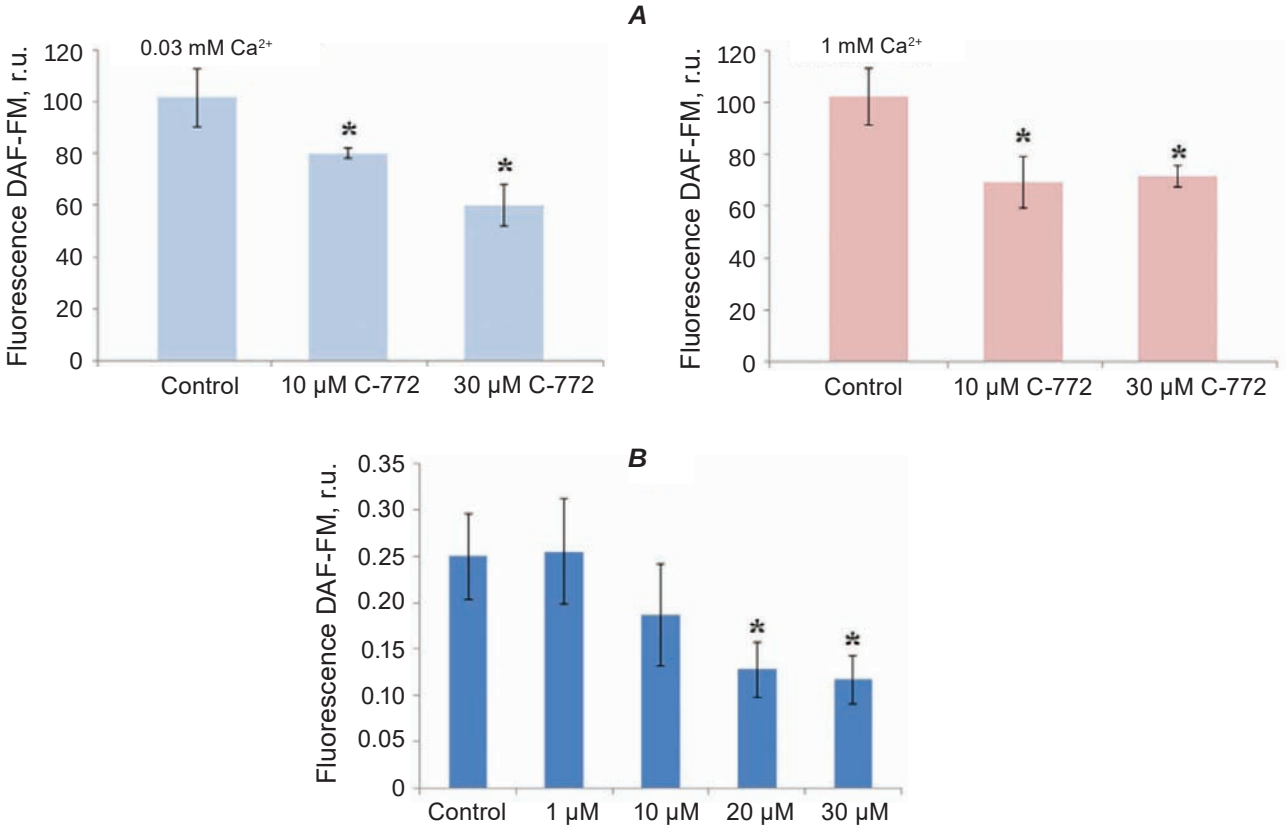

*Fig. 6. Effect of calix[4]arene C-772 on NO synthesis in myocytes (**A**) and isolated mitochondria (**B**). *P < 0.05 vs control; n = 5*

ted with Ca^{2+}-dependent processes and the functional state of the respiratory chain, the observed inhibition may reflect modulation of mitochondrial Ca^{2+} handling and/or redox-dependent regulation of NO synthase activity.

Nitric oxide is known to induce relaxation of uterine smooth muscle in both non-pregnant and pregnant states [18, 19, 26]. This effect is mediated, at least in part, by activation of the guanylate cyclase–cGMP signaling pathway and the subsequent reduction in intracellular Ca^{2+} levels. Consistently, reduced NO production has been associated with increased contractile activity in various animal models, including rats and primates [27, 28]. In this context, the inhibition of NO synthesis observed in the present study may contribute to enhanced myometrial contractility.

Calcium uptake into the mitochondrial matrix occurs via the mitochondrial calcium uniporter (MCU), a well-characterized system in myometrial mitochondria. This energy-dependent transport is driven by the high negative membrane potential of the inner mitochondrial membrane (approximately −180 mV) and proceeds via an electrophoretic mechanism [29, 30].

As shown in Fig. 7, mitochondrial Ca^{2+} accumulation under energizing conditions (in the presence of succinate and Mg-ATP^{2-}) is inhibited by calix[4]arene C-772, with a pronounced effect observed at 10 μM.

This inhibition of mitochondrial Ca^{2+} uptake is consistent with the observed decrease in NO production, given the known dependence of mitochondrial NO synthase activity on Ca^{2+} availability. Therefore, suppression of Ca^{2+} accumulation by C-772 may contribute to reduced NO synthesis in myocytes. At the cellular level, impaired mitochondrial Ca^{2+} sequestration is expected to increase cytosolic Ca^{2+} concentration. In turn, this may enhance contractile activity of the myometrium, providing a mechanistic link between the effects of C-772 on mitochondrial function, NO production, and smooth muscle contraction. The biochemical data obtained suggest that calix[4]arene C-772 may affect contractile activity of uterine smooth muscle at the tissue level. To test this, its effect (10 μM) on spontaneous contractions of rat myometrium was examined, followed by multiparametric analysis of contraction–relaxation cycles [23].

Application of C-772 increased contractile activity, with the amplitude of spontaneous contractions rising to 186.8 ± 9.8% of control (n = 3, $P < 0.05$), without significant changes in basal tone during the first 30 min of exposure and with no effect on contraction frequency (Fig. 8). Amplitude-independent parameters of the uterine cycle (duration of contraction and relaxation phases, cycle duration, inter-contraction intervals, activity index, and asymmetry) remained unchanged, whereas contractile indices expressed in Montevideo and Alexandria units, which depend on contraction amplitude, were significantly increased.

All general time parameters of myometrial contractile activity remained unchanged in the presence of C-772 (Fig. 9, *A*). In particular, the duration

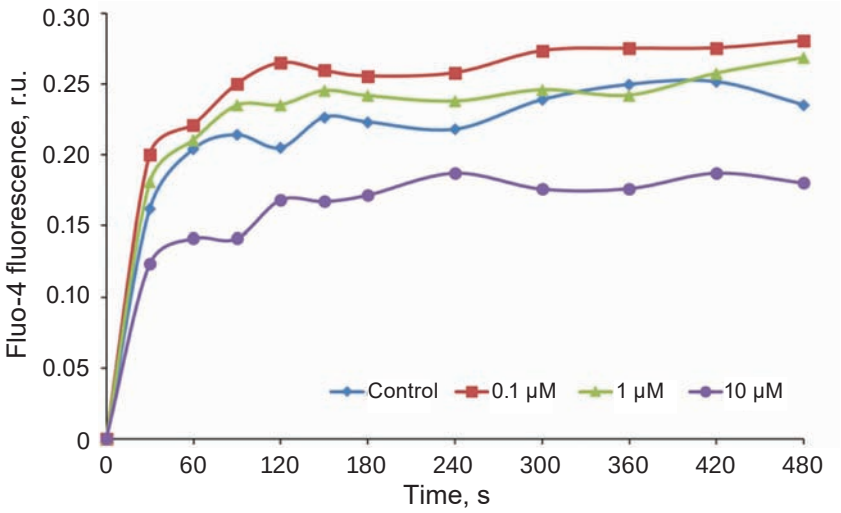

Fig. 7. Time-dependent effects of calix[4]arene C-772 (0.1, 1, 10 μM) on Ca2+ accumulation in isolated mitochondria. Representative experiment (n=5)

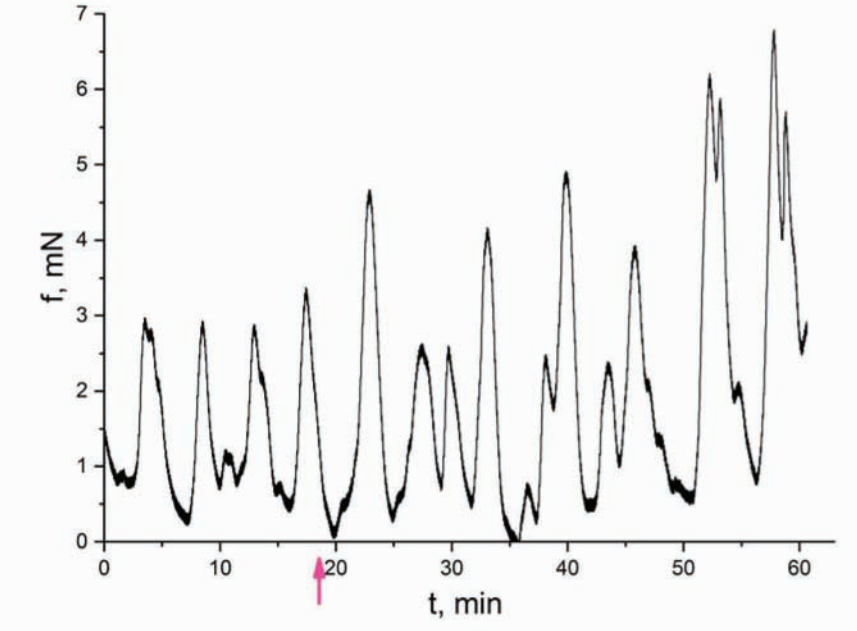

Fig. 8. Representative mechanogram of spontaneous contractions of rat uterine smooth muscle before and after addition of calix[4]arene C-772 (10 μM). The arrow indicates the time of compound application

of the contraction phase, relaxation phase, uterine cycle, and pauses between individual contractions averaged 122.0 ± 8.4%, 96.9 ± 5.9%, 106.1 ± 7.6%, and 107.4 ± 5.0% of control values, respectively (in all cases $n = 3$, $P > 0.05$). The asymmetry coefficient (ratio of contraction to relaxation phase duration) also remained at the control level, averaging 122.9 ± 10.0% ($n = 3$, $P > 0.05$; Fig. 9, *B*), while the contraction activity index showed a tendency to increase (158.3 ± 12.2%, $n = 3$, $P = 0.09$). At the same time, due to the pronounced increase in the amplitude of spontaneous contractions, calix[4]arene C-772 enhanced the overall efficiency of myometrial contractile activity, as evidenced by significant increases in contractile indices expressed in Montevideo (157.9 ± 7.3%, $n = 3$, $P < 0.05$) and Alexandria (169.6 ± 8.2%, $n = 3$, $P < 0.05$) units (Fig. 9, *C*, *D*).

The method of complex mechanokinetic analysis was applied to individual contraction–relaxation cycles. In the presence of 10 μM C-772, a significant increase in force parameters was observed: F_C and F_R reached 192.0 ± 10.8% and 184.7 ± 9.8% of control, respectively ($n = 3$, $P < 0.01$; Fig. 10, *A*). In contrast, time parameters (τ_0, τ_C, τ_R) remained unchanged (116.4 ± 7.7%, 122.3 ± 12.7%, and 114.5 ± 6.1% of control, respectively; $n = 3$, $P > 0.05$; Fig. 10, *B*). At the same time, velocity parameters (V_C and V_R) increased significantly and to a comparable extent, reaching 161.7 ± 7.6% and 169.2 ± 8.8% of control values ($n = 3$, $P < 0.05$; Fig. 10, *C*). A similar trend was observed for impulse parameters: I_0, I_C, and I_R increased to 226.3 ± 14.5%, 250.2 ± 19.3%, and 219.8 ± 13.6% of control, respectively ($n = 3$, $P < 0.05$; Fig. 10, *D*).

Thus, the effect of C-772 on myometrial contractions is primarily associated with changes in force-related parameters, without significant alterations in the temporal characteristics of contraction–relaxation cycles.

Photon correlation spectroscopy is an effective method for characterizing the size of spherical particles in solution and allows determination of their hydrodynamic diameter (characteristic size) under direct experimental conditions [24]. Changes in myocyte volume during contractile activity have been linked to alterations in sarcolemmal structures, cytoskeletal elements, and proteins of the contractile apparatus [31-33]. Most non-adherent myocytes exhibit a rounded or near-spherical shape, corresponding to a state of minimal free energy, which enables the use of this method to assess changes in their hydrodynamic diameter [34].

The classical uterotonic agent oxytocin [35] induced a decrease in the characteristic size of myocytes (Fig. 11). In contrast, the Na^+,K^+-ATPase inhibitor ouabain [36] increased their hydrodynamic diameter (Fig. 11), consistent with tensometric data showing a reduction in contractile activity of uterine

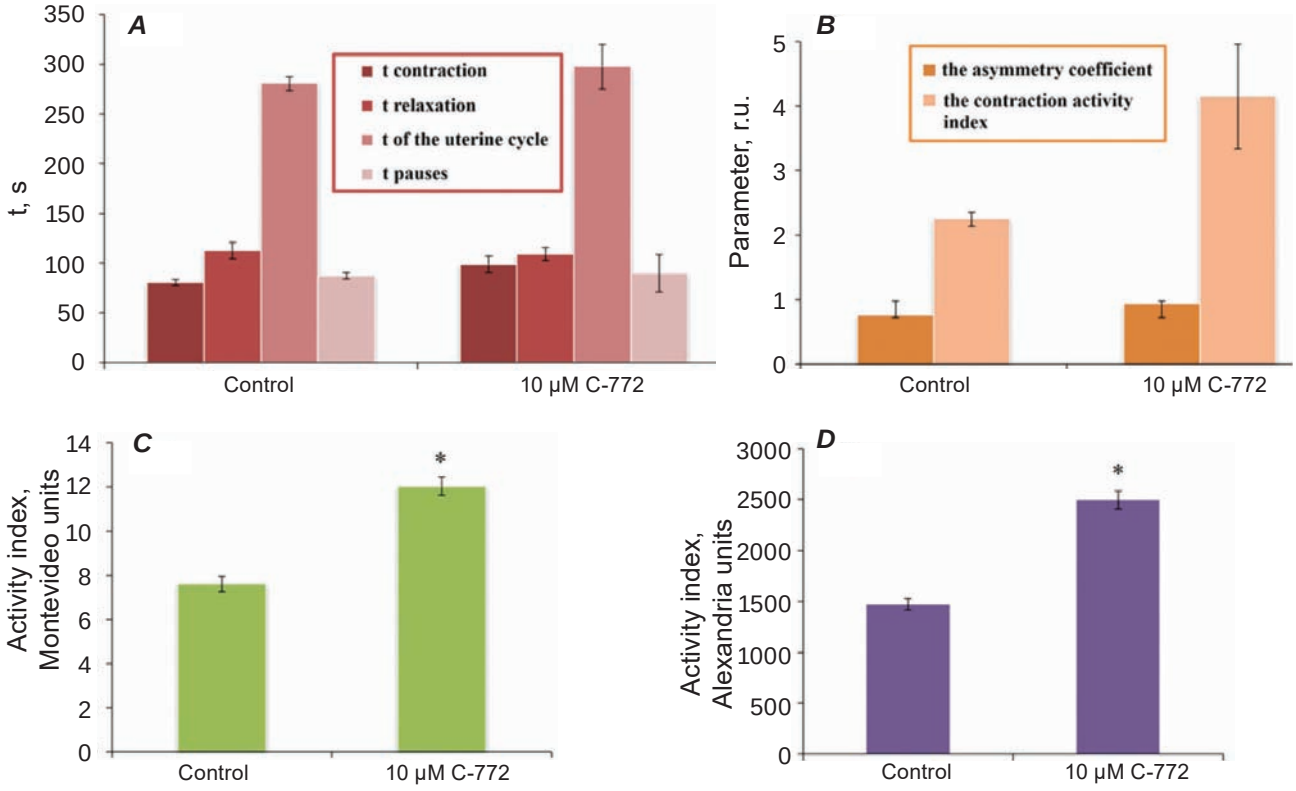

Fig. 9. Parameters of spontaneous contractile activity of rat myometrium in control and under the action of calix[4]arene C-772 (10 μM): ***A*** *– duration of uterine cycles (t of the uterine cycle), duration of pauses between cycles (t pauses), contraction phase (t contraction), and relaxation phase (t relaxation);* ***B*** *– asymmetry coefficient and contraction activity index;* ***C*** *– contractile index in Montevideo units;* ***D*** *– contractile index in Alexandria units. *P ≤ 0.05 vs control; n = 3*

smooth muscle under its action [37]. Calix[4]arene C-772 also decreased the hydrodynamic diameter of isolated myocytes, in agreement with the results of tensometric experiments. Notably, the effect of C-772 exceeded that of oxytocin at comparable concentrations (Fig. 11).

The observed changes in hydrodynamic diameter may be related to cytoskeletal rearrangements induced by modulators of contractile activity. It has been shown that myometrial contraction in pregnant women is accompanied by a decrease in myocyte volume. In addition, plasma membrane Cl^- channels, which are regulated by cell volume, have been implicated in the control of contractile activity [38, 39]. Thus, the obtained data are consistent with tensometric results indicating enhanced contractile activity of myocytes under the action of calix[4]arene C-772.

Taken together, the results suggest that the increase in contractile activity of rat myometrium induced by C-772 is, at least in part, associated with its effects on key biochemical processes in myocytes, including mitochondrial Ca^{2+} transport and NO synthesis. These findings indicate that C-772 can be used as a tool for studying the regulation of mitochondrial function and smooth muscle contraction. Considering the low toxicity of calix[4]arenes and the feasibility of their targeted synthesis, C-772 and its derivatives may represent promising candidates for further investigation as modulators of uterine contractility.

Limitations. The limitation of this study may be mitochondrial localization of calix[4]arene was demonstrated using the fluorescent conjugate C-1308 rather than calix[4]arene C-772 itself, and since C-1308 differs from C-772 in molecular structure, size, and charge distribution, the inferred mitochondrial localization of C-772 relies on the assumption that both compounds share a comparable subcellular distribution.

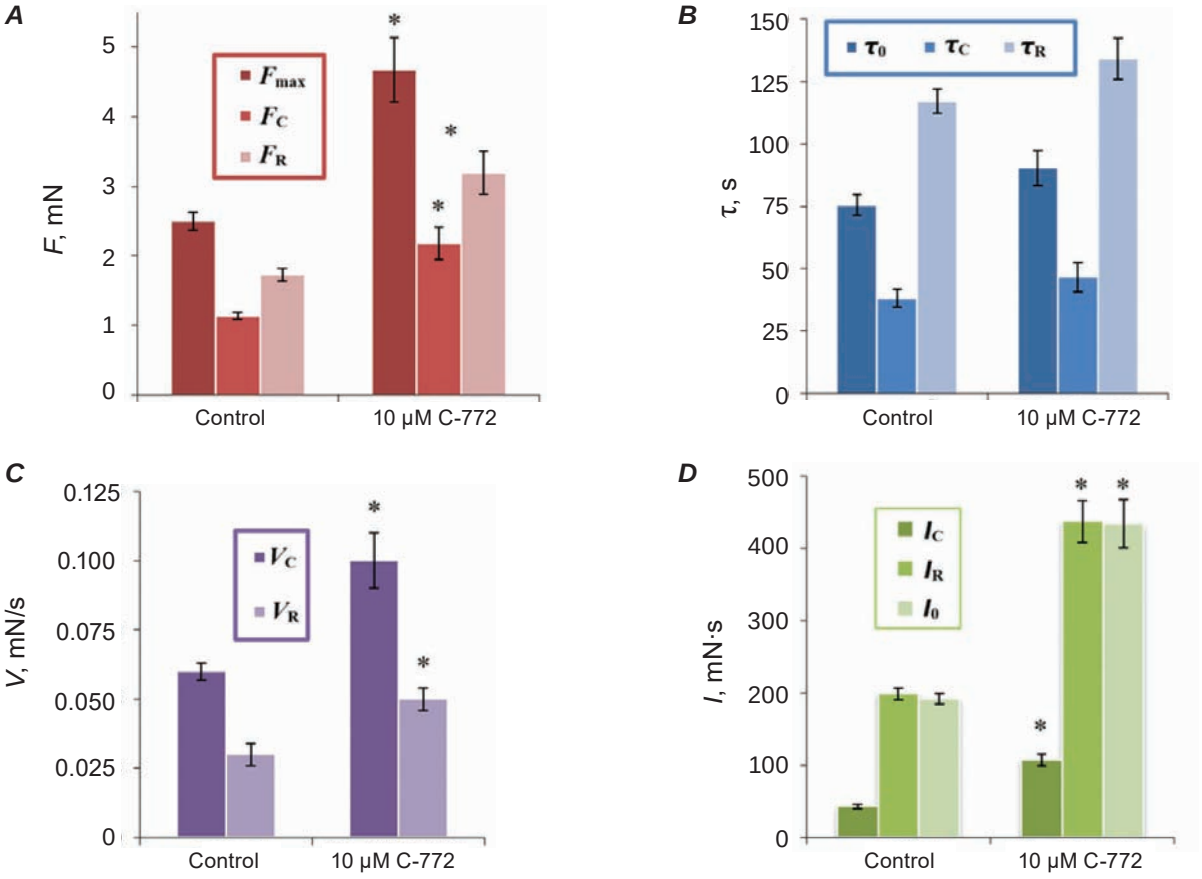

Fig. 10. Mechanokinetic parameters of spontaneous contractile activity of rat myometrium in control and after incubation with calix[4]arene C-772 (10 μM): ***A*** *– force parameters (F_{max}, F_C and F_R);* ***B*** *– time parameters (τ_0, τ_C and τ_R);* ***C*** *– velocity parameters (V_C and V_R);* ***D*** *– impulse parameters (I_0, I_C and I_R). *P < 0.05 vs control; n = 3*

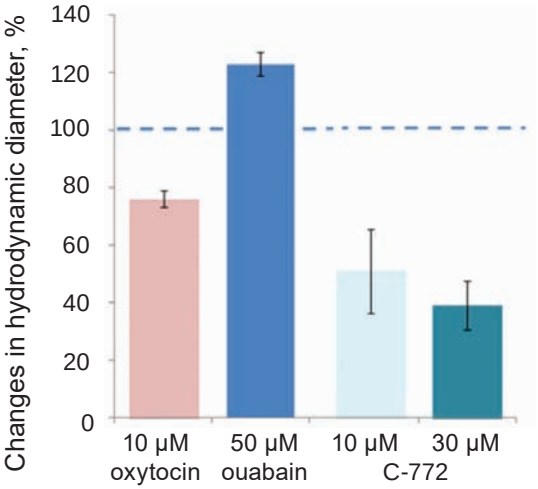

Fig. 11. Effects of calix[4]arene C-772 (10, 30 μM) and reference modulators of myometrial contractility on the hydrodynamic diameter of isolated myocytes. Values are expressed relative to control (100%), n = 5

Conclusions.

1. Calix[4]arene C-772 penetrates myocytes and interacts with mitochondria, as supported by its association with cardiolipin and mitochondrial localization of the calix[4]arene–FITC conjugate C-1308

2. C-772 (10 μM) modulates mitochondrial function by inhibiting Ca^{2+} accumulation and reducing NO production, indicating its impact on Ca^{2+}-dependent mitochondrial processes.

3. At the tissue level, C-772 enhances contractile activity of rat myometrium by increasing the amplitude of spontaneous contractions without altering their frequency or temporal characteristics.

4. Mechanokinetic analysis revealed that C-772 selectively increases force, velocity, and impulse parameters of contraction–relaxation cycles, while time-related parameters remain unaffected.

5. The decrease in hydrodynamic diameter of myocytes under C-772 is consistent with enhanced

contractile activity and supports the functional effects observed in tissue preparations.

6. Taken together, the effects of C-772 on mitochondrial Ca^{2+} handling, NO production, and contractile parameters indicate that it acts as a regulator of Ca^{2+}-dependent smooth muscle function. This compound may therefore serve as a useful tool for studying mitochondria-mediated control of myometrial contractility and as a basis for further development of uterotonic agents.

Author contributions. ISF – investigation, writing – review and editing, visualization; OVT – methodology, investigation; YVD – conceptualization, writing – original draft, supervision; HVD – methodology, formal analysis, investigation, visualization; MVR – Software, investigation; RVR – investigation, formal analysis; SOK – resources, funding acquisition.

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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Ca^{2+}-ЗАЛЕЖНІ МЕХАНІЗМИ ВПЛИВУ СПЕЦИФІЧНОГО ЩОДО МІТОХОНДРІЙ КАЛІКС[4]АРЕНУ С-772 НА СКОРОТЛИВУ АКТИВНІСТЬ ГЛАДЕНЬКОГО М'ЯЗА МАТКИ

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Вступ. Ідентифікація нетоксичних екзогенних сполук, здатних вибірково впливати на внутрішньоклітинні Ca^{2+}-залежні процеси та скоротливу активність гладеньких м'язів, залишається важливим завданням молекулярної біотехнології. Макроциклічні сполуки, а саме калікс[4]арени, вважаються перспективними модуляторами клітинних функцій. Системи транспорту Ca^{2+} плазматичної мембрани та саркоплазматичного ретикулума, а також АТРазна активність голівки міозину, є відносно нечутливими до калікс[4]арену С-772 (5,11-ди(трифторметил(фенілсульфоніл

іміно)метиламіно)-17,23-ди-трет-бутил-25,26-дипропокси-27,28-дигідроксикалікс[4]арен) у діапазоні його мікромолярних концентрацій, що дало можливість припустити вплив цієї сполуки на Ca^{2+}-залежні процеси мітохондрій. **Мета.** Метою дослідження було з'ясувати вплив калікс[4]арену С-772 на транспорт Ca^{2+} в мітохондріях гладенького м'яза матки, синтез оксиду азоту в міоцитах та скоротливу активність смужок міометрія. **Методи.** Експерименти проводили на ізольованих мітохондріях, міоцитах та смужках міометрія невагітних щурів лінії Вістар із використанням методів конфокальної мікроскопії, спектрофлуориметрії, протокової цитометрії та механокінетичного аналізу. **Результати.** Було показано, що калікс[4]арен С-772 взаємодіє з кардіоліпіном, яким збагачена внутрішня мітохондріальна мембрана. З використанням сполуки С-1308 – калікс[4]арен-FITC-кон'югату – та специфічного до мітохондрій зонду Mitotracker Orange CM-H_2TMRos продемонстровано можливість проникнення в клітину та взаємодії калікс[4]арену з субклітинними структурами. С-772 (10 мкМ) пригнічував синтез оксиду азоту та зменшував накопичення Ca^{2+} в ізольованих мітохондріях. За такої ж концентрації С-772 збільшував амплітуду спонтанних скорочень гладенького м'яза матки щурів. Механокінетичний аналіз показав збільшення параметрів сили, швидкості та імпульсу циклів скорочення-розслаблення. Крім того, обробка С-772 зменшувала характеристичний діаметр міоцитів матки у суспензії. **Висновки.** Ці результати вказують на те, що калікс[4]арен С-772 у мікромолярних концентраціях може бути ефективним модулятором функціональної активності мітохондрій та скоротливої активності гладеньких м'язів матки.

Ключові слова: мітохондрії, калікс[4]арен, гладенькі м'язи, міометрій, кальцій, кардіоліпін, оксид азоту.

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