EXPERIMENTAL WORKS

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KINETIC REGULARITIES OF THIACALIX[4]ARENE C-1087 INHIBITORY EFFECT ON THE ACTIVITY OF Mg^{2+}-DEPENDENT Ca^{2+}-TRANSPORTING ATP HYDROLASE IN THE PLASMA MEMBRANE OF SMOOTH MUSCLE CELLS

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The experiments with the suspension of plasma membranes of myometrium cells, treated with 0.1% digitonin solution, were used to study kinetic regularities of the inhibitory effect of tetrakis-N(phenylsulfonyl)-trifluoroacetamidine-thiacalixarene (C-1087) on the activity of Ca^{2+},Mg^{2+}-ATPase. The studies demonstrated the impact of C-1087 on the cumulative effect and the maximal velocity of ATP hydrolysis. No effect of C-1087 on the affinity between Ca^{2+},Mg^{2+}-ATPase, and ATP, affinity and cumulative effect of Ca ions and activation coefficient for Mg ions was revealed. A considerable decrease in the maximal velocity of ATP hydrolysis evidenced a complete non-competitive mechanism of inhibiting Ca^{2+},Mg^{2+}-ATPase activity with thiacalix[4]arene C-1087. Computer simulation demonstrated that thiacalix[4]arene C-1087 inhibiting effect on Ca^{2+},Mg^{2+}-ATPase may be conditioned by the cumulative effect of four spatially oriented N-sulfonylamidine groups on the upper rim of its macrocyclic platform.

K e y w o r d s: Ca^{2+},Mg^{2+}-ATPase, plasma membrane, smooth muscle cell, myometrium, thiacalix[4]arenes, computer simulation, docking.

The changes in the concentration of the intracellular Ca^{2+} form the basis for the contractile activity of smooth muscles. In the state of dormancy, the concentration of Ca ions in a smooth muscle cell ($[Ca^{2+}]_i$) is approximately 100 nM; during the excitation, it increases up to 1 μM (and even higher), which ensures muscle contraction. It is the extracellular Ca^{2+} (the approximate concentration value $[Ca^{2+}]_e$ – 1 mM) that plays a relevant role in the activation of the contractile function of smooth muscles (the value of Gibbs free energy ΔG in the case of transsarcolemmal calcium gradient, aimed into the cell, is $\Delta G = RT\ln\{[Ca^{2+}]_e/[Ca^{2+}]_i\}+2F\Delta\Psi \approx 40$ kilojoule/mol (ΔΨ – membrane potential, T – absolute temperature (°K), R – universal constant, F – Faraday constant)); due to the abovementioned transsarcolemmal calcium gradient, there is a passive intake of Ca^{2+} into cells during the excitation. The relaxation of muscle tension is related to the reverse decrease in Ca^{2+} concentration down to the initial level of 100 nM.

Ca^{2+}, Mg^{2+}-ATPase of the plasma membrane (PM) fulfills the function of Mg^{2+}-ATP-dependent calcium pump, which uses the energy released during Mg^{2+}-dependent hydrolysis of ATP, to pump Ca^{2+} from the cell against the concentration gradient between the extra- and intracellular medium [1, 2]. It transports one Ca ion using the hydrolysis of one ATP molecule [3]. In the unexcited cell, Ca^{2+}-pump compensates for the passive "basal" flow of Ca^{2+}, directed into myocytes, and in the excited cell – removes Ca^{2+} from the cells, thus controlling the relaxation of myocytes and maintaining the physiological concentration of Ca ions in the state of dormancy [4].

Taking the abovementioned into consideration, the search for compounds which would allow for targeted changing the activity of Ca^{2+}-pump of PM in case of any impairments in its functioning due to pathological states would be a promising task. From this standpoint, noteworthy molecular platforms are calixarenes – macrocyclic oligomers of phenols with three-dimensional "cup"-like structure. Due to practically unlimited possibilities of the chemical modification of the macrocyclic frame using functional groups of various nature, regulation of hydrophilic-hydrophobic balance of the molecule within a broad range, low toxicity, no immunogenicity, (thia)calixarenes are actively investigated as nanovectors for targeted delivery of the API of medical preparations, as modulators of enzymatic activity, biomimetics of receptors and enzymes, molecular nanoplatforms for the design of biologically active compounds, etc. [5-8].

In our previous study [9], we demonstrated that 5,11,17,23-tetra-N-phenylsulfonyltrifluoroacetamidine-25,27-dihexyloxy-26,28-dihydroxy-thiacalixarene (titer C-1087) in the concentration of 100 μM effectively (85 % as compared to the control value) inhibited the enzymatic activity of Ca^{2+},Mg^{2+}-ATPase in the PM fraction of uterus myocytes, practically not affecting the activity of Mg^{2+}-independent Ca^{2+}-dependent ATPase, Na^{+},K^{+}-ATPase, and Mg^{2+}-ATPase, localized in the same membrane structure.

To investigate the mechanism of the inhibitory effect of C-1087 on the transporting Ca^{2+},Mg^{2+}-ATPase, in this work, we studied kinetic regularities of the inhibition process.

Materials and Methods

A. Synthesis and structure of compounds under investigation

^{1}H, ^{13}C, ^{19}C NMR spectra were registered using Varian VXR 400 and Bruker Advance DRX 500 spectrometers, the working frequencies are indicated in the descriptions of spectra, the chemical shifts (δ) are expressed in parts per million (ppm) regarding the signal of the solvent or the standard (TMS), the constants of spin-spin interaction (J) are presented in hertz (Hz). Compounds **2**, **1087**, and **1145** were synthesized in the anhydrous organic solvents under argon. Compound **M1** [10] was synthesized previously.

*5,11,17,23-tetranitro-25,27-dihexyloxy-26,28-dihydroxythiacalixarene **2**.* To suspension of tetranitrothiacalixarene **1** [11] (1.7 g, 2.51 mmol) and potassium carbonate (2.75 g, 19.9 mmol) in DMF (25 ml) the hexyliodide (4.15 g, 3 ml, 19.6 mmol) was added under stirring. Reaction was stirred at 75°C for 48 h. The 1 M solution of sulfuric acid (30 ml) was added after cooling. After 10-15 min light suspension was decanted off, sticky residue was suspended in chloroform (30 ml) and washed with 1 M hydrochloric acid (30 ml), brine (30 ml) and evaporated on rotor evaporator (15 mm Hg, 50°C). Dark-yellow solid was washed with diethyl ether (20 ml) and was filtrated. Obtained yellow crude thiacalixarene **2** was purified by column chromatography (SiO_2, 40-60 μm, $CHCl_3$-CH_3OH, 100:1, v/v, R_f 0.5). Slightly yellow crystalline compound, yield 1.8 g (84.8%) M. p. 242-244°C. ^{1}H NMR (399.98 MHz, $CDCl_3$) δ: 0.93 (t, J = 6.86 Hz, 6H, O-CH_2-CH_2-CH_2-$(CH_2)_2$-CH_3), 1.40 (m, 8H, O-CH_2-CH_2-CH_2-$(CH_2)_2$-CH_3), 1.58 (m, 4H, O-CH_2-CH_2-CH_2-$(CH_2)_2$-CH_3), 2.03 (m, 4H, O-CH_2-CH_2-CH_2-$(CH_2)_2$-CH_3), 4.39 (t, J = 6.86 Hz, 4H, O-CH_2-CH_2-CH_2-$(CH_2)_2$-CH_3), 7.99 (s, 4H, ArH (OHexyl)), 8.12 (s, 2H, OH), 8.67 (s, 4H, ArH(OH)). ^{13}C NMR (125.70 MHz, $CDCl_3$) δ: 14.02, 22.51, 25.28, 29.79, 31.45, 79.11, 121.89, 129.29, 131.69, 132.62, 140.19, 143.96, 162.87, 164.26.

*5,11,17,23-tetraamino-25,27-dihexyloxy-26,28-dihydroxythiacalixarene **3**.* To the suspension of tetranitrothiacalixarene **2** (1.3 g, 1.54 mmol) in 2-propanol (40 ml) the hydrazine hydrate (4 ml) was added. The resulted mixture was heated up to complete dissolution of solid phase. Aqueous suspension of Raney nickel (10% by weight, 3 ml) was added to the obtained solution. Reaction mixture was refluxed till its yellow color disappearance. In the case of the end of gas evolution, the hydrazine hydrate (2 ml) was added and the reaction was continued. Gas evolution (N_2) was checked by the gas bubble counter filled with water. After cooling, reaction mixture was filtrated through silica gel. Silica gel was washed by warm DMF (20 ml). The filtrates were combined, solvent was removed on the rotor evaporator (15 mm Hg, 65°C). A toluene (20 ml) was added to light brown oily residue and resulted mixture was again evaporated on rotor evaporator (15 mm Hg, 65°C). Tetraaminothiacalixarene **3** was dried in vacuum (12 mm Hg, 80°C) for 4 hours. Gray-green solid compound, yield 0.92 g (82.6%) M. p. 205-208°C. ^{1}H NMR (399.98 MHz, DMSO-D_6) δ: 0.87 (brs, 6H, O-CH_2-CH_2-CH_2-$(CH_2)_2$-CH_3), 1.35 (brs, 8H, O-CH_2-CH_2-CH_2-$(CH_2)_2$-CH_3), 1.53 (brm, 4H, O-CH_2-CH_2-CH_2-$(CH_2)_2$-CH_3), 1.82 (brm, 4H, O-CH_2-CH_2-CH_2-$(CH_2)_2$-CH_3), 4.24 (brt, 4H, O-CH_2-CH_2-CH_2-$(CH_2)_2$-CH_3), 4.71 (brs, 8H, NH_2),

6.28 (s, 4H, ArH(OHexyl)), 6.88 (s, 4H, ArH(OH)), 7.06 (s, 2H, OH). ^{13}C NMR (125.70 MHz, DMSO-d_6) δ: 13.98, 22.12, 25.08, 29.53, 31.17, 74.99, 120.72, 121.77, 121.79, 128.15, 140.79, 145.63, 148.39, 148.88.

5,11,17,23-tetra-N-phenylsulfonyltrifluoroacetamidine-25,27-dihexyloxy-26,28-dihydroxy-thiacalixarene ***C-1087****.* To the suspension of tetraaminothiacalixarene 3 (0.435 g, 0.6 mmol) and triethylamine (0.265 mg, 2.61 mmol) in tetrahydrofurane (THF, 30 ml) was added dropwise the solution of N-phenylsulfonyltrifluoroacetylimidoylchloride [12] (0.815 g, 2 mmol) in THF (15 ml). The reaction mixture was mixed at 70°C for 8 h. After cooling, the precipitate was filtered off. The residue (a light orange solid foam) obtained after solvent removal from filtrate by the rotor evaporator (15 mm Hg, 50°C) was re-precipitated by water (2 ml) from ethanol (9 ml). The obtained precipitate was filtered and dried in the vacuum (12 mm Hg) for 3 h, then dissolved in hot benzene (5 ml) and precipitated by heptane (15 ml). The obtained precipitate of crude C-1087 was purified by column chromatography (SiO_2, 40-60 μm, chloroform-methanol, 100:1, v/v, R_f 0.6). The yield – 0.24 g, 24%, light yellow solid, M. p. 185-187°C, 1H NMR (400.13 MHz, CD_3OD) δ: 0.93 (t, J = 6.78 Hz, 6H, O-CH_2-CH_2-CH_2-$(CH_2)_2$-CH_3), 1.41 (m, 8H, O-CH_2-CH_2-CH2-$(CH_2)_2$-CH_3), 1.60 (m, 4H, O-CH_2-CH_2-CH_2-(CH2)2-CH_3), 1.93 (m, 4H, O-CH_2-CH_2-CH_2-$(CH_2)_2$-CH_3), 4.37 (brt, 4H, O-CH_2-CH_2-CH_2-$(CH_2)_2$-CH_3), 4.71 (brs, 8H, NH_2), 6.95-7.4 (brs, 4H, ArH(OHexyl)), 7.54 (m, 12H, SO_2PhH-para and meta), 7.65 (t, J = 7.03 Hz, 4H SO_2PhH-meta), 7.85 (d, J = 7.28 Hz, 4H SO_2PhH-orto), 7.91 (s, 4H, ArH(OH)). ^{19}F NMR (470.26 MHz, DMSO-d_6) δ: -65.67, -65.55. ^{13}C NMR (125.70 MHz, DMSO-d6) δ: 13.90, 22.06, 24.81, 29.05, 31.38, 73.63, 115.45. 117.73, 120.76, 125.80, 125.93, 127.64, 129.18, 132.40, 132.58, 142.63, 142.75, 156.44, 163.81.

25,27-dihexyloxy-26,28-dihydroxythiacalixarene ***C-1145****.* Triphenylphosphine (0.655 g, 2.5 mmol) and n-hexanol (0.215 g, 2.1 mmol) were added to the suspension of thiacalixarene **4** (0.495 g, 1 mmol) in THF (10 ml) and mixed for 15 min. Diethyl azodicarboxylate (0.435 g, 2.5 mmol) was added dropwise to this suspension. The reaction mixture was mixed for 12 h at room temperature. Light precipitate was filtered off and filtrate was evaporated on rotor evaporator (15 mm Hg, 50°C). The oily orange-brown residue was washed with methanol (15 ml). The obtained precipitate of crude thiacalixarene C-1145 was filtered, dried on air to the constant weight, and purified by column chromatography (SiO_2, 40-60 μm, hexane-ethyl acetate, 4:1, v/v, R_f 0.62). A colorless crystalline compound. Yield 0.51 g (77%), M. p. 202-204°C, ^{1}H NMR (399.98 MHz, $CDCl_3$) δ: 0.91 (t, J = 6.86 Hz, 6H, O-CH_2-CH_2-CH_2-$(CH_2)_2$-CH_3), 1.38 (brs, 8H, O-CH_2-CH_2-CH_2-$(CH_2)_2$-CH_3), 1.55 (brm, 4H, O-CH_2-CH_2-CH_2-$(CH_2)_2$-CH_3), 1.98 (m, 4H, O-CH_2-CH_2-CH_2-$(CH_2)_2$-CH_3), 4.35 (t, J = 6.86 Hz, 4H, O-CH_2-CH_2-CH_2-$(CH_2)_2$-CH_3), 6.48 (t, J = 7.77 Hz, 2H, ArH (OHexyl)), 6.81 (t, J = 7.77 Hz, 2H, ArH(OH)), 6.82 (d, J = 7.77 Hz, 4H, ArH(OHexyl)), 7.35 (s, 2H, OH), 7.61 (t, J = 7.38 Hz, 4H, ArH(OH)).

B. Biochemical studies

The biochemical studies were conducted at the Muscle Biochemistry Department of the Palladin Institute of Biochemistry, the NAS of Ukraine (headed by S. O. Kosterin, a full member of the NAS of Ukraine).

Preparative biochemistry. The PM fraction of uterine SM cells was isolated from the porcine myometrium as described before [13, 14].

The protein content in the membrane fraction was determined by the method of M. Bredford [15] using the reaction with Coomassie reagent – G250.

Enzymological studies. The total ATPase activity was determined in the PM fraction of myometrium cells at 37°C in the standard medium (volume – 0.4 ml), containing (mM): 3 ATP, 3 $MgCl_2$, 0.95 $CaCl_2$, 25 NaCl, 125 KCl, 1 EDTA, 20 Hepes-tris-buffer (pH 7.4), 1 NaN_3, 1 ouabain, 0.1 μM thapsigargin, and 0.1 % digitonin. The calculations, made in the MAXCHEL program, demonstrated that under these physical, chemical, and concentration conditions of the incubation medium, the concentration of free Ca (proper Ca^{2+}) was approximately 1 μM. While studying the impact of different concentrations of Ca ions on the activity of Ca^{2+}, Mg^{2+}-ATPase, the required Ca^{2+} concentrations were also preset by computerized computations using the abovementioned program. The amount of membrane fraction protein in the probe – 20–30 μg. The incubation time – 5 min. The enzymatic reaction was initiated by the introduction of the aliquot (50 μl) of PM suspension to the incubation medium, and terminated by the introduction of 1 ml of "stop"-solution to the incubation mixture as follows: 1.5 M acid sodium acetate, 3.7% formaldehyde, 14 % ethanol, 5% TCA, pH 4.3 (at 8°C). The amount of P_i reaction product was determined by the method of W. Rathbun et V. Betlach [16].

Ca^{2+}, Mg^{2+}-ATPase activity was estimated by the difference between the values of ATPase activi-

ties in the presence and the absence of exogenous Ca ions (against the background of 1 mM EDTA – specific chelating agent for Ca ions) in the incubation medium. In the sarcolemma of the porcine myometrium, the average enzymatic activity of Ca^{2+},Mg^{2+}-ATPase was 3.4 ± 0.3 μmol P_i/mg of protein per one hour ($M \pm m$; $n = 7$).

The basal Mg^{2+}–ATPase activity was determined in the PM fraction of myometrium cells at 37°C in the medium (volume – 0.4 ml), containing (mM): 1 ATP, 3 $MgCl_2$, 125 NaCl, 25 KCl, 1 EDTA, 20 Hepes-tris-buffer (pH 7.4), 1 NaN_3, 1 ouabain, 0.1 μM thapsigargin and 0.1% digitonin. The "basal" Mg^{2+}-ATPase activity was estimated as a difference between the amount of P_i, which was formed in the incubation medium in the presence and the absence of PM fraction with the consideration of the amendment for the content of endogenous P_i in the membrane preparation.

Na^+,K^+-ATPase activity was determined in the same medium and estimated by the difference between the values of ATPase activities in the absence and the presence of 1 mM ouabain.

In our experiments, the average value of activities of Na^+,K^+-ATPase and "basal" Mg^{2+}-ATPase of PM was 10.2 ± 0.7 and 18.1 ± 1.2 μmol P_i/mg of protein per one hour, respectively ($n = 7$).

It should be noted that the PM of uterine myocytes was found to also contain Ca^{2+}-ATPase, the properties of which differed from Ca^{2+},Mg^{2+}-ATPase, since its activity was manifested in the presence of millimolar concentrations of Ca^{2+} and ATP in the incubation medium against the background of the absent Mg ions [17, 18]. Ca^{2+}-ATPase was of low affinity to the activating cation – the constant of activation with Ca ions for K_{Ca} was 1 mM [18]. The low-affinity Mg^{2+}-independent Ca^{2+}–ATPase activity was determined in the PM fraction of myometrium cells at 37°C in the medium (volume – 0.4 ml), containing (mM): 1 ATP, 3 $CaCl_2$, 125 NaCl, 25 KCl, 1 EDTA, 20 Hepes-tris-buffer (pH 7.4), 1 NaN_3, 1 ouabain, 0.1 μM thapsigargin, and 0.1 % digitonin. The mentioned Ca^{2+}-ATPase activity was estimated as a difference between the amount of P_i, formed in the incubation medium in the presence and the absence of PM fraction with the consideration of the amendment for the content of endogenous P_i in the membrane preparation. In the sarcolemma of the porcine myometrium, the enzymatic activity of low-affinity Mg^{2+}-independent Ca^{2+}-ATPase of PM was 12.7 ± 2.0 μmol P_i/mg of protein per one hour ($n = 7$).

In the experiments on the impact of different concentrations of C-1087 (1–100 μM) on Ca^{2+},Mg^{2+}-ATPase activity, the above-described standard incubation medium was used with the addition of the aliquot of C-1087 solution in the relevant concentration. The experiments involved the use of the concentrated (20 mM) solution of C-1087 in DMSO, which was further diluted with water.

Kinetic estimates. While studying the concentration dependences of the effect of thiacalixarene on the enzymatic activity, the values of inhibition coefficients $I_{0.5}$ and Hill coefficients n_H were estimated using the linearized charts of Hill according to the equation $\lg[(A_{max}-A)/A] = -n_H \lg I_{0.5} + n_H \lg[\text{C-1087}]$, where A_{max} and A – enzymatic activities in the absence ("zero point") and in the presence of thiacalixarene in the incubation medium in the concentration of C-1087.

The values of the apparent constants of the activation of Ca^{2+},Mg^{2+}-ATPase with Ca K_{Ca} ions, and Hill coefficients n_{Ca} were calculated using the linearized charts of Hill according to the equation $\lg[(A_{max} - A)/A] = n_{Ca}\lg K_{Ca} - n_{Ca}\lg[Ca^{2+}]$, where A_{max} and A – enzymatic activities under optimal and active concentration of calcium Ca^{2+} in the incubation medium.

The apparent maximal initial velocity V_{max} of the ATP hydrolysis reaction was determined using the charts, built in the coordinates of Lineweaver-Burk according to the equation $1/V = K_{Ca}/[Ca^{2+}]V_{max} + 1/V_{max}$ where V – enzymatic activity in the presence of Ca in the incubation medium in the concentration of Ca^{2+}.

Computer simulation. The geometry of three-dimensional structure of thiacalix[4]arenes C-1087 and C-1145 was optimized by the method of energy minimization. The minimization was conducted by the method of MMFF94 (Merck Molecular Force Field) with the consideration of electrostatic and Van der Waals interactions under the following conditions: ascension slope – 100; step size of ascension slope – 0.002 nm; conjugated gradient of ascension steps – 10; the size of the conjugated gradient of ascension steps – 0.002 nm. The minimization was performed in ChemOffice program using MMFF94 method. The optimized structures were used while docking with the enzyme in AutoDock program, version 4.2 [19].

The optimization of the geometry (energy minimization) of the interaction between thiacalixarenes and Ca^{2+},Mg^{2+}-ATPase, and the molecular dy-

namics was done in Chimera program [20]. The optimal results were selected both by geometric indices for the ligand position in the center of binding the complex "thiacalixarene – Ca^{2+},Mg^{2+}-ATPase" and by the energy indices, using the functions of estimating the binding energy in the "receptor-ligand" complex, "built" into the docking program. A series of complexes with the least free energy of interaction was chosen in the simulation of the interaction between thiacalixarene and Ca^{2+},Mg^{2+}-ATPase. Then the optimal geometry of the formed complexes was estimated, and the energy-wise beneficial location of the ligand in the space of Ca^{2+},Mg^{2+}-ATPase was determined. Chimera program was used to analyze the results and to prepare the figures using the simulation results.

The stability of binding and the conformational changes in the investigated complex were studied by the method of molecular dynamics (MD). MD was conducted with the consideration of electrostatic and Lennard-Jones interactions, under the following conditions: interval of a step of calculation – 1.0 fs; frame interval – 10 fs; ending after 10,000 steps; velocity of heating/cooling – 1 ccal/atom/ps; Andersen barostat – 1.0132 bar, deoxidation time – 1.5 ps; Nose thermostat – 398 K, deoxidation time – 0.2 K/ps. The field of force Amber ff14SB was used. The method of estimating the charge of Zn atoms – AM1 BCC.

The spatial structure of Ca^{2+},Mg^{2+}-ATPase of PM (SR) with 6llE identifier in the database of these proteins (RSCB Protein Data Bank) was used in the study.

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 MS Excel 2018.

Reagents. The following reagents were used in the experiments: ATP, Hepes, ouabain, thapsigargin, Hoechst, fluo-4 AM, collagenase, poly-L-lysine (Sigma, USA), tris-hydroxymethyl-aminomethane (Reanal, Hungary), digitonin (Merck, Germany), EDTA (Fluka, Switzerland), oxytocin (Gideon Richter, Hungary). Other reagents were analytically and chemically pure, produced in Ukraine.

Results and Discussion

Thiacalix[4]arene C-1087 is synthesized by three-step process, starting from tetranitrothiacalixarene **1** [12] according to Fig. 1. The first stage is regioselective distal 1,3-dialkylation of tetranitrothiacalixarene **1** with hexyliodide in DMF in the presence of potassium carbonate as a base. The second stage is reduction of tetranitrodihexyloxythiacalixarene **2** with hydrazine hydrate – Raney nickel system. The third stage, is the reaction of aminothiacalixarene **3** with N-phenylsulfonyltrifluoroacetimidoylchloride in the presence of triethylamine that lead to obtain the desired thiacalixarene **C-1087**.

Thiacalixarene **C-1145** is synthesized by the Mitsunobu reaction of tetrahydroxycalixarene **4** and hexanol in the presence of triphenylphosphine and diethyl ether of azodicarboxylic acid according to Fig. 2. The reaction takes place regioselectively, with the formation of the 1,3-dialkylation product.

Dihexyloxythiacalixarenes **2**, **3**, **C-1087** and **C-1145** are exist in solutions in the cone-like conformation with syn-orientation of four phenyl rings of the macrocycle. This is confirmed by chemical shifts of hexyl substituents OCH_2 protons at 4.24–4.39 ppm in ^1H NMR spectra. In case 1,3-alternate conformation which characterized *syn-anti*-orientation of adjacent phenyl rings pairs of the macrocycle corresponding chemical shifts must be shifted to strong field δ 3.81-3.86 ppm as a result their shielding by phenyl rings of macrocyclic skeleton [21-23].

In our previous studies [10], we showed that C-1087 in the concentration of 100 μM effectively (by 85% as compared to the control) inhibited the enzymatic activity of Ca^{2+},Mg^{2+}-ATPase in PM of uterine myocytes. It inhibited the enzymatic activity of Ca^{2+},Mg^{2+}-ATPase of sarcoplasmic reticulum much less effectively (by 34% as compared to the control) [10]. At the same time, C-1087 in this concentration practically did not impact the enzymatic properties of Na^+,K^+-ATPase, "basal" Mg^{2+}-ATPase, and Ca^{2+}-ATPase of PM: the corresponding activities were 90.1 ± 0.7; 95.3 ± 1.0 and 89.1 ± 0.8% regarding the control value *(n* = 5).

Thus, C-1087 selectively (at the PM level) inhibited the activity of Ca^{2+},Mg^{2+}-ATPase of PM, not affecting the activities of Na^+,K^+-ATPase, basal Mg^{2+}-ATPase, and Ca^{2+}-ATPase in PM.

We also studied the concentration dependence of the inhibitory effect of C-1087 (10^{-8}–10^{-4} M) on the activity of Ca^{2+},Mg^{2+}-ATPase in PM. The estimated value of the inhibition coefficient $I_{0.5}$ was 9.4 ± 0.6 μM, the value of Hill coefficient n_H was 0.58 ± 0.03 (n = 5).

To determine the role of the constituent fragments of C-1087 molecule in inhibiting Ca^{2+},Mg^{2+}-ATPase, two model compounds were studied:

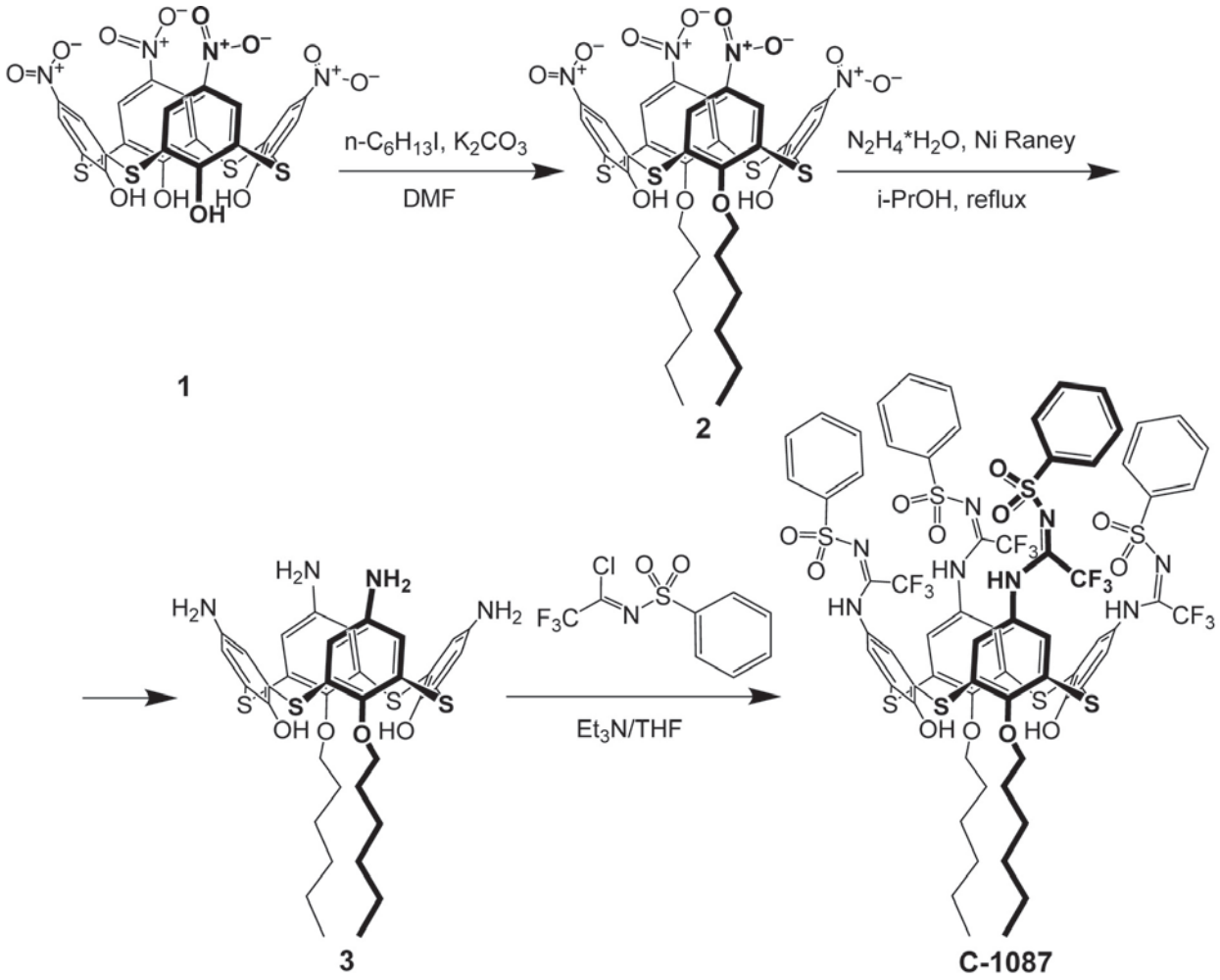


Fig. 1. The synthesis of thiacalixarene C-1087

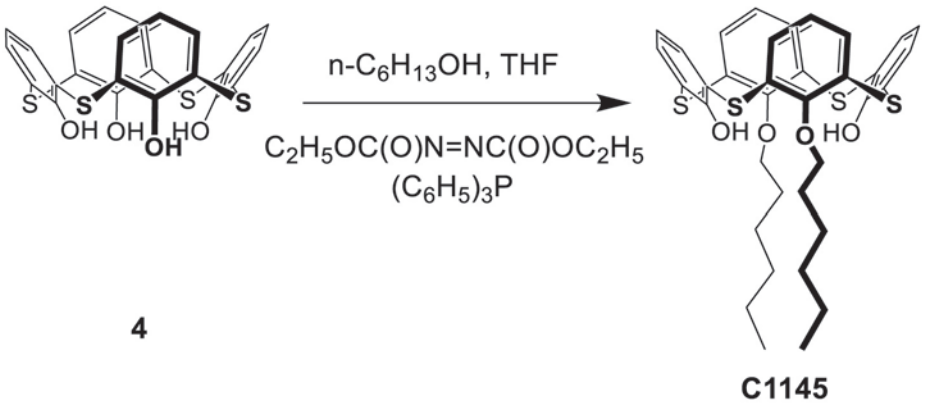


Fig. 2. The synthesis of thiacalixarene C-1145

25,27-dihexyloxy-26,28-dihydroxythiacalixarene C-1145 (calixarene bowl) and N-(para-ethoxy)phenyl-N'-phenylsulfonyl-trifluoromethylacetamidine M-1 (Fig. 4) [11] as a functional amidine constituent.

It was found that the molecular bowl of C-1145 in the concentration of 100 μM inhibited Ca^{2+},Mg^{2+}-ATPase activity only by 14.0 ± 1.2%) (n = 5). The model compound M-1 in the concentration of 100 and 200 μM inhibited Ca^{2+},Mg^{2+}-ATPase activity only by 9.3 ± 2.4 and 11.3 ± 1.4% respectively, as compared to the control value (n = 5) (Fig. 3).

Therefore, the inhibitory effect of C-1087 was achieved by the synergetic effect of previously spatially organized of sulfonylamidine groups on the calixarene platform. For the purposes of further kinetic interpretation of the inhibitory effect of C-1087, we studied its impact on the nature of dependence between the activity of Ca^{2+},Mg^{2+}-ATPase, and the concentrations of ATP and Ca and Mg ions.

The increase in ATP concentration in the incubation medium in the range from 0.01 to 3 mM in the absence of C-1087 led to the increase in the enzymatic activity of Ca^{2+},Mg^{2+}-ATPase (Fig. 5, *A*, control) under the fixed concentration of $MgCl_2$ (3 mM) in the incubation medium. Hill's method was used to calculate the apparent K_{ATP} and Hill coefficient n_H, which were 56.3 ± 4.3 μM and 1.3 ± 0.1, respectively (n = 5) (Fig. 5, *B* and *C*).

We studied the impact of C-1087 ([C-1087] = 1, 10, 30, 60, and 100 μM) on the dependence of the enzyme activity on ATP concentration (Fig. 5,

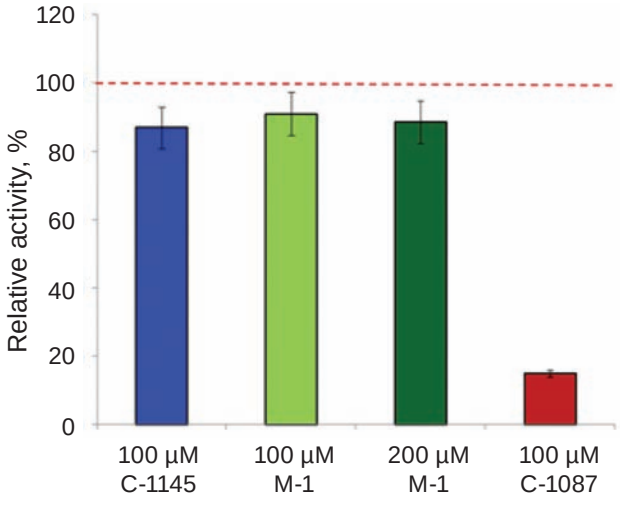
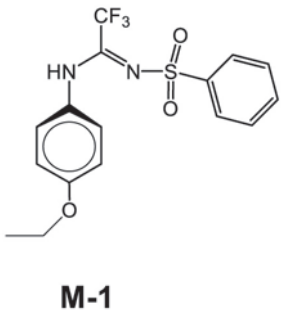


Fig. 3. The activity of Ca^{2+},Mg^{2+}-ATPase at the effect of C-1087, C-1145 and M-1 (n = 5). The values of enzymatic activities in the absence of C-1087 in the incubation medium are accepted as 100%



M-1

Fig. 4. The structural formula of M-1 functional amidine constituent

A). In all cases, a monotonic decrease in the activity of Ca^{2+},Mg^{2+}-ATPase was observed, while the dependence of enzymatic activity on ATP showed a character similar to the corresponding control dependence without thiacalix[4]arene C-1087. But there was a decrease in the plateau level of activity with increasing concentration of thiacalix[4]arene.

Using the obtained dependences, we estimated the maximal initial velocity V_{max} of ATP hydrolysis reaction, which was catalyzed by Ca^{2+},Mg^{2+}-ATPase in PM in the absence and the presence of C-1087 (Fig. 5, *B*). As seen in the chart, C-1087 reduced V_{max} of the reaction which demonstrated the decrease in the enzyme turnover number under its effect. We also estimated the apparent constants of K_{ATP} and Hill coefficients n_H, in the absence and in the presence of C-1087 (Fig. 5, *C*). The presented results demonstrated non-competitive effect of C-1087 regarding ATP. It can be assumed that the substrate center of Ca^{2+},Mg^{2+}-ATPase, and the binding site of C-1087 are located in different areas of the enzyme surface.

In our further experiments, we studied the dependence of the activity of Ca^{2+},Mg^{2+}-ATPase on the concentration of Ca^{2+} in the incubation medium in the presence of ([C-1087] = 1, 10, 30, 60 and 100 μM). In this case, we calculated the concentration of Ca ions, taking into consideration EDTA concentration and its affinity to Ca^{2+}. The enzymatic activity of Ca^{2+},Mg^{2+}-ATPase of PM in myometrium increased along with the concentration of Ca ions from 100 to 1000 nM, but decreased monotonously along with the increase in the concentration of the inhibitor (Fig. 6, *A*).

We estimated the kinetic parameters of the activation with Ca ions and the impact of C-1087 on them (Fig. 6, *B* and *C*). The value of V_{max} was decreasing along with the increase in the concentra-

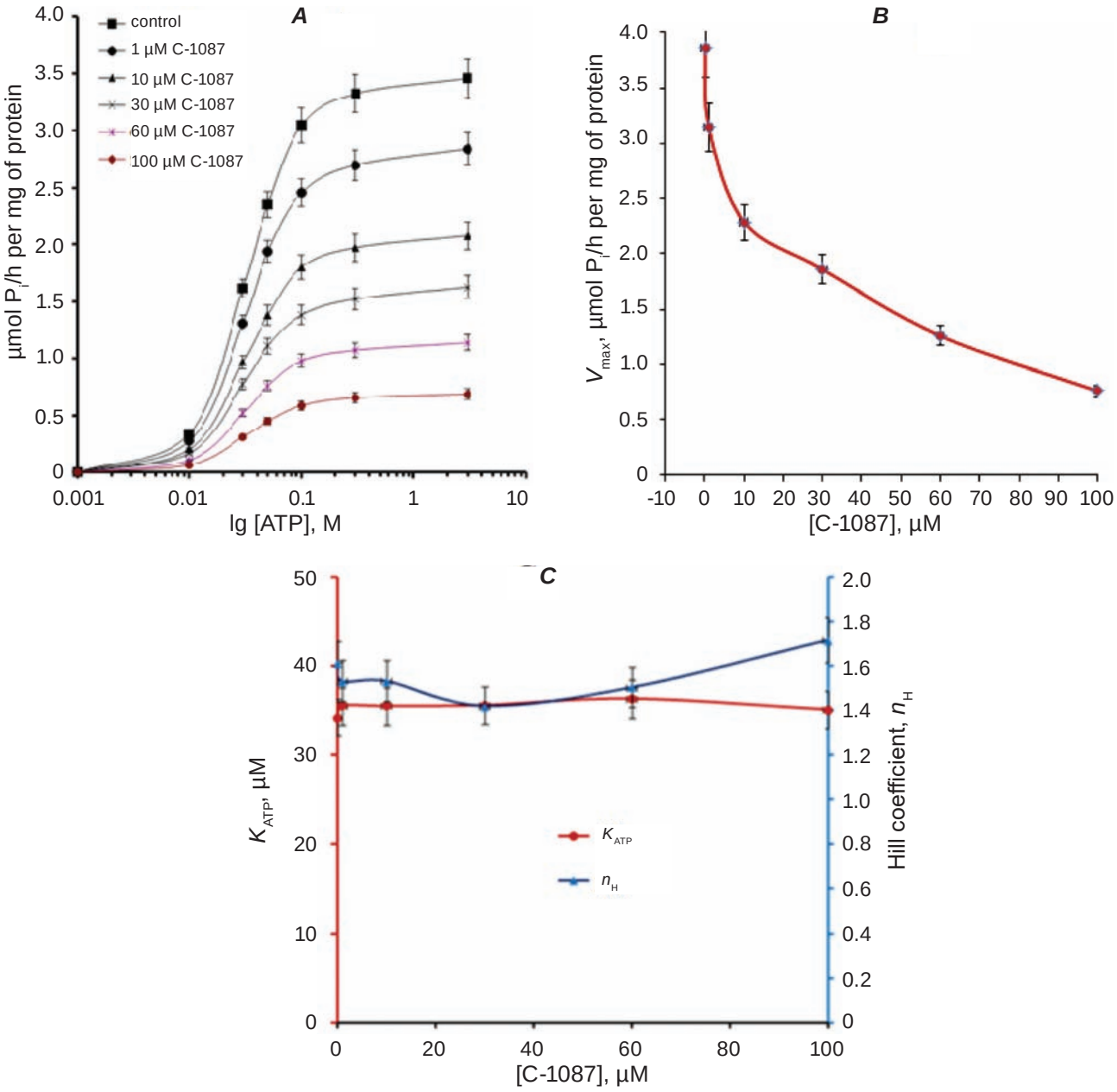


Fig. 5. ***A****. The impact of C-1087 concentration on the dependence of Ca^{2+},Mg^{2+}-ATPase activity from ATP concentration (n = 5).* ***B****. The impact of C-1087 concentration on the maximal initial velocity V_{max} of ATP hydrolysis reaction by ATP, catalyzed by Ca^{2+},Mg^{2+}-ATPase (n = 5).* ***C****. The impact of C-1087 concentration on kinetic parameters of the ATP hydrolysis reaction by ATP (apparent K_{ATP} and Hill coefficient n_H), catalyzed by Ca^{2+},Mg^{2+}-ATPase (n = 5)*

tion of C-1087 (Fig. 6, *B*). The value of the activation coefficient for Ca^{2+},Mg^{2+}-ATPase K_{Ca} in the absence of C-1087 was 190 ± 11 nM, the value of Hill's coefficient n_H – 2.1 ± 0.1 (*M* ± *m*; *n* = 5). The introduction of C-1087 (100 μM) into the incubation medium insignificantly (up to 235 ± 12 nM) increased the constant of the activation with Ca ions. Here the value of Hill's coefficient increased up to 2.4 ± 0.1 (*n* = 5).

Thus, in the concentration range up to 100 μM, C-1087 had practically no impact on the affinity of Ca^{2+},Mg^{2+}-ATPase to Ca ions and on the cumulative effect of the enzyme activation with these ions.

The relevance of Mg^{2+} for metabolism is explained by its properties as a promoter of the structure of protein macromolecules, substrate-binding ions, and electron transporters. There are many

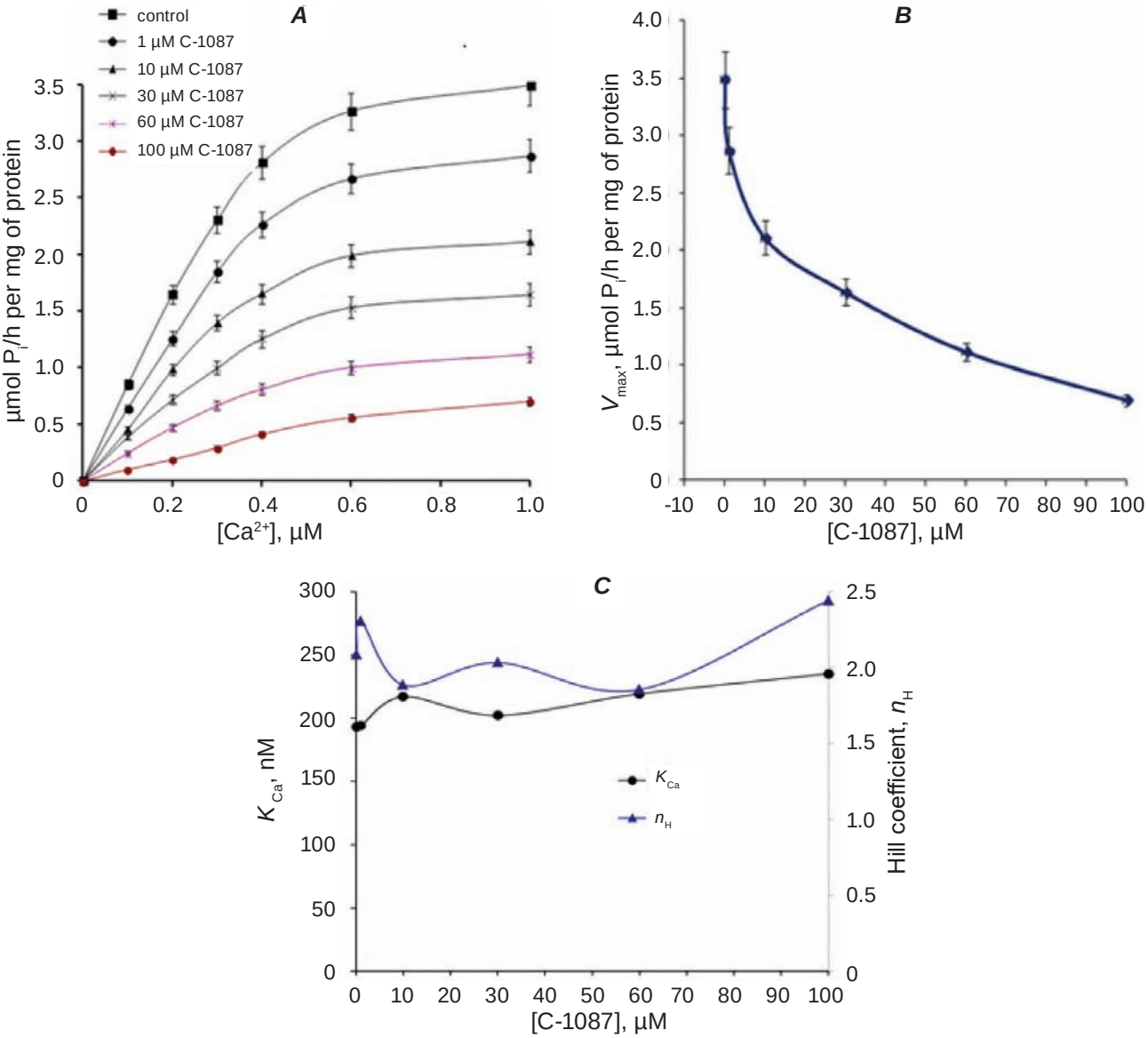


Fig. 6. ***A****. The impact of C-1087 concentration on the dependence of Ca^{2+},Mg^{2+}-ATPase activity on the concentration of Ca ions (n = 5).* ***B****. The impact of C-1087 concentration on the maximal initial velocity V_{max} of ATP hydrolysis reaction by Ca^{2+}, catalyzed by Ca^{2+},Mg^{2+}-ATPase (n = 5).* ***C****. The impact of C-1087 concentration on kinetic parameters of the ATP hydrolysis reaction by Ca^{2+} (activation coefficient K_{Ca} and Hill coefficient n_H), catalyzed by Ca^{2+},Mg^{2+}-ATPase (n = 5)*

known Mg^{2+}-dependent enzymes, where the role of Mg^{2+} is not limited to substrate activation but is related to the formation of the active (catalytic) center. However, the most widely-known role of Mg^{2+} is the one it plays in the formation of the chelate complex with ATP – the substrate of adenosine triphosphatase reactions. It is believed that Mg^{2+} ions interact with phosphate-charged groups of ATP, polarize them and raise the reactive ability of the system, facilitating the nucleophilic attack on the terminal phosphate residue of ATP [1, 24].

We studied the impact of C-1087 ([C-1087] = 1, 10, 30, 60, and 100 μM) on the concentration dependence of ATP-hydrolase activity on $MgCl_2$ (Fig. 7, *A*). The enzymatic activity of Ca^{2+},Mg^{2+}-ATPase increased along with the increase in the concentration of $MgCl_2$ from 0.1 to 3 mM on condition of fixed concentrations of ATP (3 mM) in the incubation medium and was monotonously inhibited along with the increase in the concentration of C-1087. The value of the apparent activation constant for Ca^{2+},Mg^{2+}-ATPase K_{Mg} was 0.7 ± 0.1 mM, the

value of Hill coefficient n_H – 1.0 ± 0.1 (n = 5). In all cases, the introduction of C-1087 into the incubation medium was accompanied by the decrease in the activity of Ca^{2+},Mg^{2+}-ATPase. The value of V_{max} was decreasing along with the increase in the concentration of C-1087 (Fig. 7, *B*). It was demonstrated that at the impact of C-1087 (up to 100 μM), there was almost no change in the activation coefficient for Ca^{2+},Mg^{2+}-ATPase by magnesium chloride. At the concentration of C-1087 of 100 μM, the value of K_{Mg} increased up to 0.8 ± 0.1 mM. Here the value of Hill coefficient increased up to 2.4 ± 0.2 (n = 5) (Fig. 7, *C*).

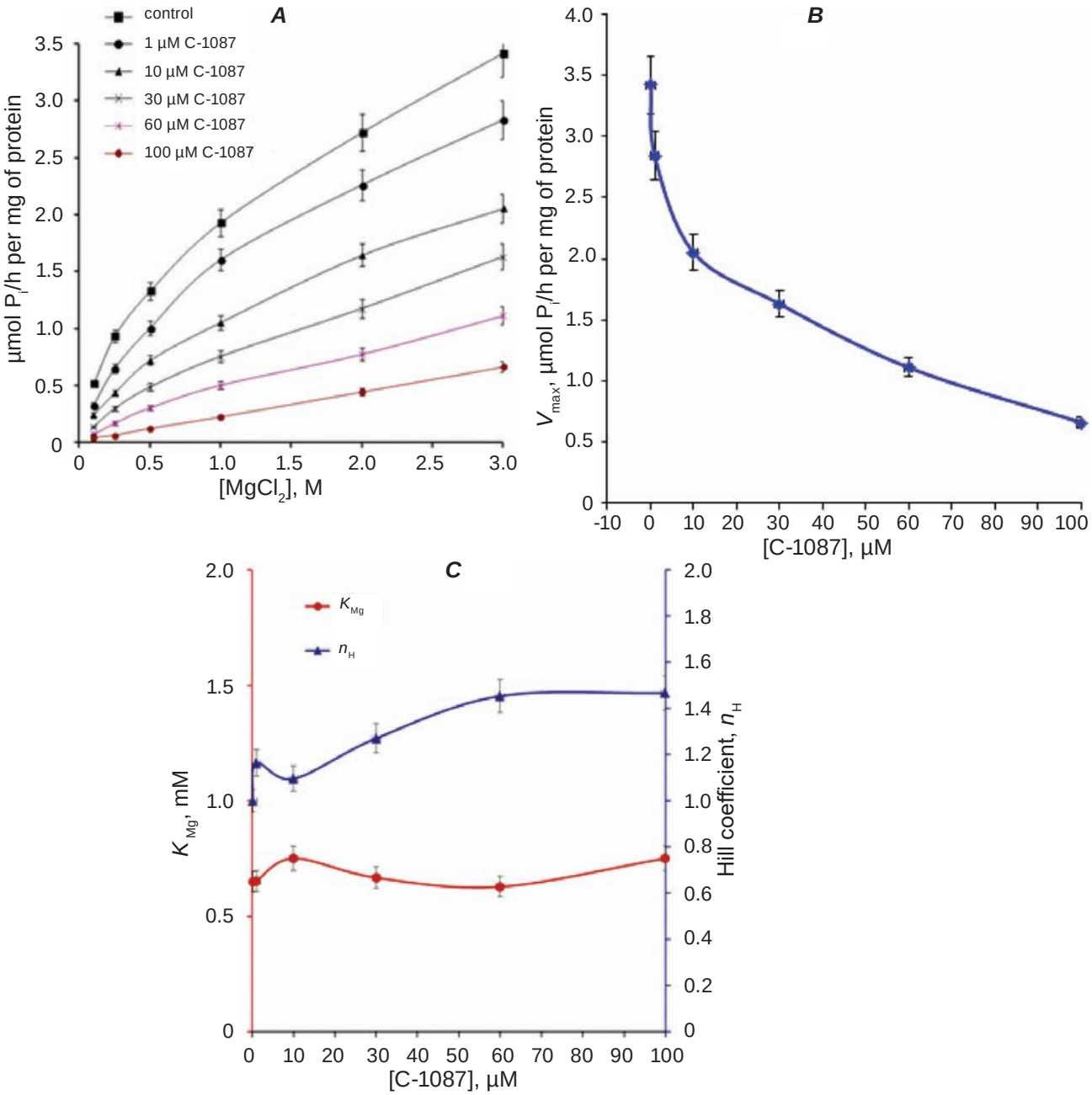


Fig. 7. ***A****. The impact of concentration C-1087 on the dependence of Ca^{2+},Mg^{2+}-ATPase activity on the concentration of $MgCl_2$ (n = 5).* ***B****. The impact of C-1087 concentration on the maximal initial velocity V_{max} of the ATP hydrolysis reaction by Mg^{2+}, catalyzed by Ca^{2+},Mg^{2+}-ATPase (n = 5).* ***C****. The impact of C-1087 concentration on kinetic parameters of the ATP hydrolysis reaction by Mg^{2+} (apparent activation constant K_{Mg} and Hill coefficient n_H), catalyzed by Ca^{2+},Mg^{2+}-ATPase (n = 5)*

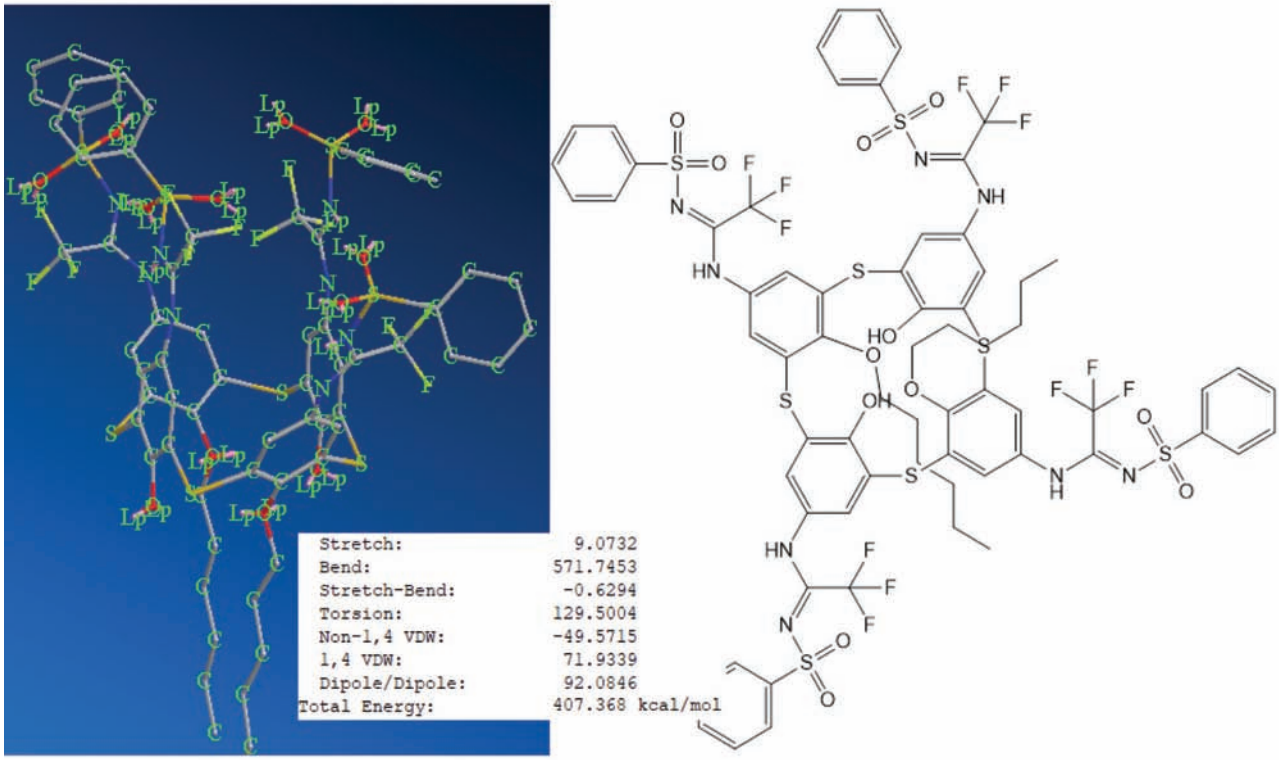


Fig. 8. Energy-wise minimized structure of thiacalix[4]arene C-1087

Thus, in the entire concentration range, C-1087 had practically no impact on the affinity of Ca^{2+},Mg^{2+}-ATPase to Mg ions and on the cumulative effect of the enzyme activation with the indicated ions.

More detailed data about the spatial structure of thiacalixarenes was obtained by the method of energy minimization, and the total energy of the molecule was 407 kcal/mol. After the "minimization", thiacalix[4]arene C-1087 had a somewhat "inverted" cone-like conformation (Fig. 8).

It required detecting the potential sites of interaction between ligands and ligand-binding sites (LBS) of Ca^{2+},Mg^{2+}-ATPase, the presence of which was confirmed by the values of the minimal binding energy and aminoacid environment of LBS. Taking into account the sizes and geometric conformation, one can expect that C-1087 and its model C-1145 (molecular bowl) will have different affinity to the sites of binding Ca^{2+},Mg^{2+}-ATPase. The types of interactions between the enzyme and C-1087 were also determined.

The exact localization of C-1087 in LBS of the enzyme is complicated due to the fact that the calcium pump is a membrane-bound enzyme, and there are literature data about rather considerable shifts of cytoplasmic domains of ATPases of P-type regarding each other in the enzymatic cycle. Thus, we determined the most probable regions of interaction between C-1087 and the enzyme, confirmed by the values of minimal binding energy and aminoacid environment of LBS.

Considering the structure, sizes, and conformation of thiacalix[4]arenes C-1087 and C-1145, they can have different affinity to the sites of binding Ca^{2+},Mg^{2+}-ATPase. The reason for it can be found in the difference in organizing the environment of ligand-binding sites for Ca^{2+},Mg^{2+}-ATPase for these thiacalix[4]arenes. Taking into consideration the structural specificities of C-1087, we analyzed the data about the binding of this ligand, obtained by the docking method. We defined possible places of coordination ("cavities") of the investigated ligands to LBS of Ca^{2+},Mg^{2+}-ATPase and their interaction with the receptor was studied, which allowed for selecting some complexes with the lowest total energy.

The docking demonstrated that C-1087 may be located in the two most probable regions of Ca^{2+},Mg^{2+}-ATPase (Fig. 9).

The first site of binding C-1087 is in the area, structurally close to the binding site with a high af-

finity to Ca^{2+}, which is in the area of the loop between the helices M4-M6 and helix M-8 (Fig. 10, *A*).

This area is a hydrophobic pocket with hydrophilic residues in the periphery. The following residues interact while binding in this area with sulfonylamidine groups of thiacalix[4]arene: Cys909, Leu912, Leu915, Asn929, Leu932, Cys937, Met940, Leu970, Leu974, Leu1021, Leu1025. The phenyl rings of calixarene bowl take part in the stacking interactions with aminoacid residues Thr905, Pro926, Trp927, Val933, Ile936 (Fig. 10, *B*).

Obviously, the binding of thiacalix[4]arene C-1087 molecule in this area may impact the conformational mobility of the enzyme. This is how the reaction cycle of the enzyme and the affinity to the ions, involved in the ATP hydrolysis reaction and transportation of Ca^{2+}, are impacted which is confirmed by the experimental data.

The relevant structural specificities of Ca^{2+},Mg^{2+}-ATPase may be described as the presence of the aminoacid residues, not directly involved in catalysis mechanisms but spatially closing the access to the center of substrate binding, close to the active center. Considering the ability of C-1087 to penetrate

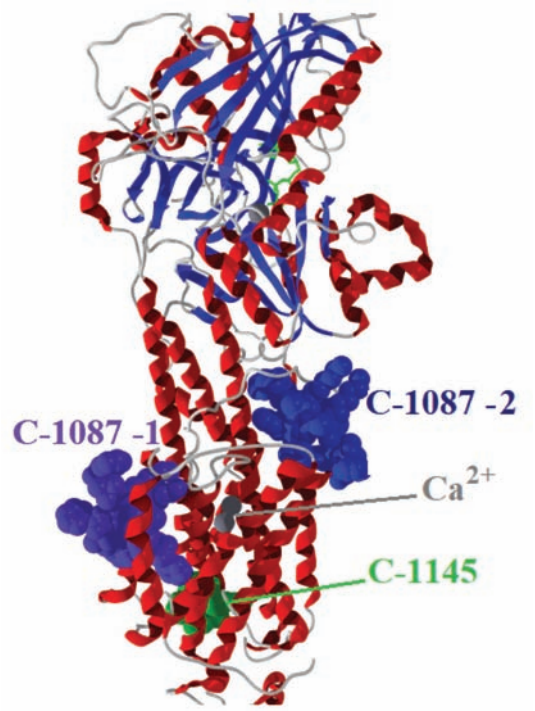


Fig. 9. The most probable sites 1 and 2 of binding ligands C-1087, C-1145 and functionally active sites of Ca^{2+},Mg^{2+}-ATPase

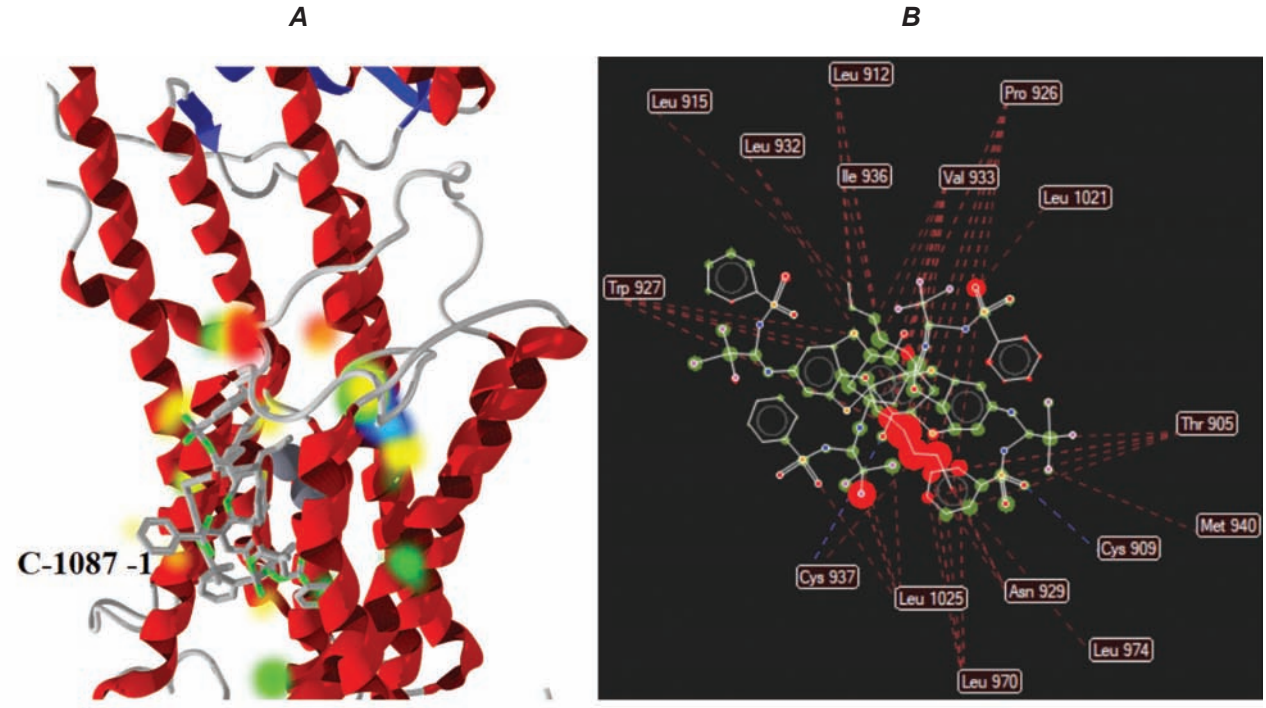


*Fig. 10. **A**. Map of different types of interactions involved in the stabilization of C-1087 with Ca^{2+},Mg^{2+}-ATPase of the plasma membrane in region 1. **B**. Interactions between C-1087 and Ca^{2+},Mg^{2+}-ATPase in the site of binding 1. H-bonds of aminoacid residues with the inhibitor are indicated with brown dotted lines. Green color – steric interactions; turquoise color – hydrogen acceptors; yellow – hydrogen donors; red-blue – electrostatic interactions*

the membrane, it is possible that it interacts with cytoplasmic fragments of the enzyme.

In the binding site 2, sulfonylamidine groups of C-1087 interact with the charged groups of aminoacids, and phenyl residues take part in hydrophobic and stacking interactions with the molecule of Ca^{2+},Mg^{2+}-ATPase (Fig. 11, *A*). A molecule of C-1087 may spatially block the nucleotide-binding center of the enzyme. The residues Arg750, Arg655 and Lys492 interact with oxygen atoms of sulfonyl groups. Gly343, Cys344, Gly749, Lys757, Lys818, Trp831 and Ala987 interact with trifluoromethyl groups. Some positively charged atoms (of nitrogen and sulfur) of the C1087 molecule interact with residues Glu825, Glu917, and Glu992. The phenyl rings of calixarene bowl of molecule C-1087 may take part in the hydrophobic and stacking interactions with the residues of aromatic aminoacids – Tyr753, Pro819, Pro820, Tyr990, and Leu991 (Fig. 11, *B*).

Thus, the docking of C-1087 to ligand-binding sites of the enzyme demonstrates that the substrate receptor complex is stabilized by hydrogen bonds, electrostatic, hydrophobic, and stacking interactions.

At the same time, the model compound – calixarene bowl C-1145 – may interact with the enzyme in the region of the accumulation of aromatic residues in the near-membrane area (the areas of α-helix: 73-84, 284-294), which is on the extracellular side. The experimentally confirmed insignificant inhibitory impact of C-1145 may be explained by the effect of C-1145 on the conformation of transmembrane helix M-8, where one of the binding sites for Ca^{2+} is located.

Therefore, the data of this work may serve as a foundation for the elaboration of effective inhibitors of Ca^{2+},Mg^{2+}-ATPase of the plasma membrane to determine the membrane mechanisms of cation exchange in smooth muscles, in particular, for the investigation of the role of the plasma membrane in ensuring the electromechanical conjugation in them, and in the regulation of ion homeostasis in smooth muscle cells. Selective inhibitors of the calcium pump in the plasma membrane may find their practical application as pharmacological means to correct intracellular concentration of Ca ions under pathological states.

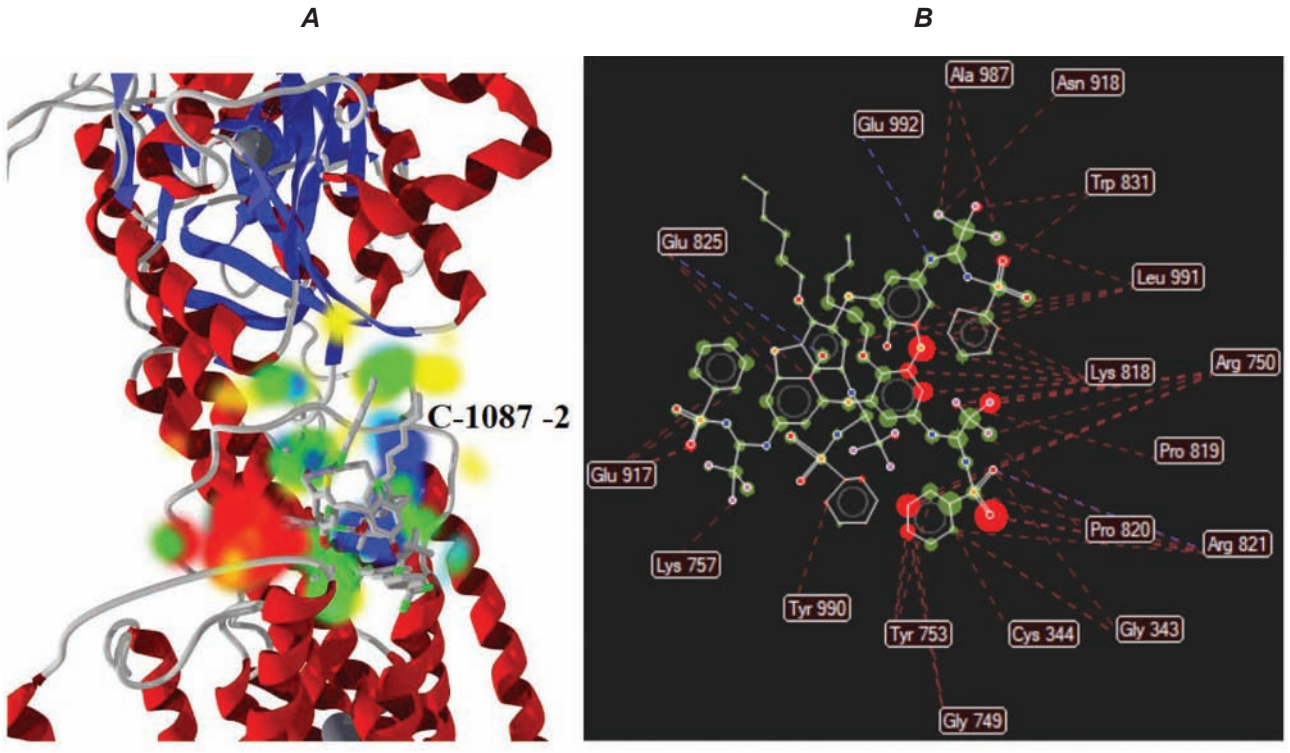


*Fig. 11. **A**. Map of different types of interactions involved in the stabilization of C-1087 with Ca^{2+},Mg^{2+}-ATPase of the plasma membrane in region 2. **B**. Interactions between C-1087 and Ca^{2+},Mg^{2+}-ATPase in the site of binding 2. H-bonds of aminoacid residues with the inhibitor are indicated with brown dotted lines. Green color – steric interactions; turquoise color – hydrogen acceptors; yellow – hydrogen donors; red-blue – electrostatic interactions*

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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КІНЕТИЧНІ ЗАКОНОМІРНОСТІ ІНГІБУЮЧОЇ ДІЇ ТІАКАЛІКС[4]АРЕНУ С-1087 НА АКТИВНІСТЬ Mg^{2+}-ЗАЛЕЖНОЇ Ca^{2+}-ТРАНСПОРТУЮЧОЇ АТР-ГІДРОЛАЗИ В ПЛАЗМАТИЧНІЙ МЕМБРАНІ ГЛАДЕНЬКОМ'ЯЗОВИХ КЛІТИН

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Суспензія плазматичних мембран клітин міометрія, оброблена 0,1% розчином дигітоніну, була використана для вивчення кінетичних закономірностей інгібуючої дії тетра-N-фенілсульфоніл трифторацетамідинтіакаліксарен (С-1087) на активність Ca^{2+},Mg^{2+}-АТРази. Дослідження продемонстрували вплив С-1087 на кумулятивний ефект та максимальну швидкість гідролізу АТР. Не виявлено впливу С-1087 на спорідненість Ca^{2+},Mg^{2+}-АТРази до АТР, а також спорідненість і кумулятивний ефект іонів Са та коефіцієнт активації іонів Са та Mg. Суттєве зниження максимальної швидкості гідролізу АТР свідчить про повний неконкурентний механізм інгібування Ca^{2+},Mg^{2+}-АТРазної активності тіакалікс[4]арену. Комп'ютерне моделювання показало, що інгібуючий вплив тіакалікс[4]арену С-1087 на Ca^{2+},Mg^{2+}-АТРазу може бути зумовлений кумулятивним впливом чотирьох просторово орієнтованих N-сульфоніламідинових груп на верхньому ободі його макроциклічної платформи.

Ключові слова: Ca^{2+},Mg^{2+}-АТРаза, плазматична мембрана, гладеньком'язові клітини, міометрій, тіакалікс[4]арени, комп'ютерне моделювання, докінг.

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