EXPERIMENTAL WORKS

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INHIBITION OF PLASMA MEMBRANE Ca^{2+},Mg^{2+}-ATPase BY CALIXARENE SULFONYLAMIDINES. STRUCTURE-ACTIVITY RELATIONSHIP

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Previously we have already shown that tetrasulfonylamidinecalixarene C-90 inhibited plasma membrane Ca^{2+},Mg^{2+}-ATPase of smooth muscle cells selectively to other ATPases of plasma membrane. To inhance the inhibitory effect of calixarenes several alkoxycalixarene sulfonylamidines structurally similar to calixarene C-90 were synthesized and their effects on the mentioned enzyme activity, the level of cytoplasmic Ca^{2+} concentration and hydrodynamic diameter of isolated smooth muscle cells were checked. It was shown that sulfonylamidino groups are crucial for Ca^{2+},Mg^{2+}-ATPase inhibition, the efficiency of inhibition depends on their quantity and spatial orientation at the upper rim of calixarene macrocycle. Introduction of phenyl or tert-butyl groups into the upper rim and of long alkyl chains into the lower rim led to only slightl increase of inhibition efficiency. The inhibitory effect of studied calixarenes on Ca^{2+},Mg^{2+}-ATPase correlated with effects on cytosolic Ca^{2+} concentration and hydrodynamic diameter of smooth muscle cells. The obtained results are important for creation of more effective and selective inhibitors of plasma membrane Ca^{2+},Mg^{2+}-ATPase as regulators of smooth muscle contractility.

K e y w o r d s: plasma membrane Ca^{2+},Mg^{2+}-ATPase, intracellular Ca^{2+} concentration; smooth muscle; calixarene sulfonylamidines.

The contraction-relaxation dysfunction of uterus smooth muscle (SM) of myometrium can usually be foundation of different pathologies such as premature labor, fetal wastage, uterine inertia, post-partum atony, uterine hypotonia, uterine bleeding, etc [1, 2]. Therefore searching of new pharmacological compounds capable to modify muscle tone is perspective for treatment of mentioned pathologies. Such muscle dysfunctions often caused by alteration in membrane-bound systems of cation transport, as it is well known that control of muscle contraction, are realized via changing of Ca^{2+} concentration, in particular by Mg^{2+}-dependent ATP-hydrolyzing cation-transporting enzymes [2, 3].

There are several ATPases in plasma membrane (PM) of SM. Ca^{2+},Mg^{2+}-ATPase of smooth muscle or PMCA (EC 7.2.2.10) is one of the principal for regulation of Ca^{2+} concentration because this enzyme maintains low cytosolic [Ca^{2+}] in relaxed cells by compensation of passive Ca^{2+} entrance to the cells and decrease the Ca^{2+} concentration after cell excitation when it extremely rises. SM has small intracellular depot of Ca^{2+}, so muscle relaxation is a result of Ca^{2+} pumping to intracellular space. Thereby PMCA plays crucial role in SM relaxation due to pumping out of Ca^{2+}.

Nowadays we have no selective inhibitors of PMCA besides so-called caloxins which are oligopeptide acting extracellularly and obtained by genome engineering method using screening a random peptide phage display library [4]. So, design of low-molecular inhibitors of PMCA which could

cross plasma membrane and enter to the cells is very perspective.

According to our previous investigations such perspective compounds are calixarenes – synthetic bowl-like shape macrocyclic oligomers of phenols and formaldehyde. Calixarenes, due to ability to give supramolecular complexes with biologically important molecules and ions, can influence on biochemical processes and therefore are considered as original molecular platforms which are perspective for designing of biologically active compounds [5, 6]. Main advantages of calixarenes are their low toxicity and immunogenicity [7]. By now numerous experimental data evidence that some calixarenes have bactericidal, antiviral, antitumoral, antithrombotic activity and other biological properties [8]. Some calixarenes are effective inhibitors and activators of enzymes [9, 10]. Calixarenes can also effect on biochemical and physicochemical properties of cell membranes [8, 11]. Thus, the potential of calixarenes as biologically active compound are not discovered fully.

Previously [12] we have already shown that tetrasulfonylamidinecalixarene C-90 inhibited PMCA (IC_{50} = 20.2 ± 0.5 μM) selectively to other ATPases of PM, but we still don't understand what chemical groups of calixarene C-90 are crucial for interaction with the molecular surface of PMCA. Such knowledge let us improve inhibitory properties of calixarenes relative to PMCA such as efficiency and selectivity.

Thus, the aim of this investigation is to determine structure-activity relationship of sulfonylamidinocalixarenes which are important for artificial ingibition of PMCA activity and therefore can increase of free [Ca^{2+}] inside muscle cells and their contraction. For this purpose we studied and compared the effect of a series of calixarenes, which were structurally similar to calixarene C-90, on PMCA activity, cytoplasmic free [Ca^{2+}] and hydrodynamic diameter of isolated smoothmuscle cells (SMCs).

Materials and Methods

Calixarenes used in research

We used mentioned below calixarenes in our ivestigation:

calixarene C-90 − 5,11,17,23-tetra(trifluoro) methyl(phenylsulfonylimino)methylamino-25,26,27,28-tetrapropoxycalix[4]arene;

calixarene C-715 −5,17-di(trifluoro)acetamido-11,23-di-tert-butyl-26,28-dihydroxy-25,27-dipropoxycalix[4]arene;

calixarene C-716 − 5,17-di(trifluoro) methyl(phenylsulfonylimino)methylamino-11,23-di-tert-butyl-26,28-dihydroxy-25,27-dipropoxycalix[4] arene;

calixarene C-772 − 5,11-di(trifluoro) methyl(phenylsulfonylimino)methylamino-17,23-di-tert-butyl-25,28-dihydroxy-26,27-dipropoxycalix[4] arene;

calixarene C-956–5,11,17,23- tetra(trifluoro) methyl(phenylsulfonylimino)methylamino-25,27-dioctyloxy-26,28-dipropoxycalix[4]arene;

calixarene C-957–5,11,17-tri(trifluoro) methyl(phenylsulfonylimino)methylamino-23-tert-butyl-26,28-dihydroxy-25,27-dipropoxycalix[4] arene;

calixarene C-960–5,17-di(trifluoro) methyl(phenylsulfonylimino)methylamino-26,28-dihydroxy-25,27-dipropoxycalix[4]arene;

calixarene C-975–5,11,17,23-tetra(trifluoro) methyl(methylsulfonylimino)methylamino-25,26,27,28-tetrapropoxycalix[4]arene;

In all experiments we used initial concentrated solutions of calixarenes (20 mM) in DMSO which were diluted with buffer solution to necessary final concentration.

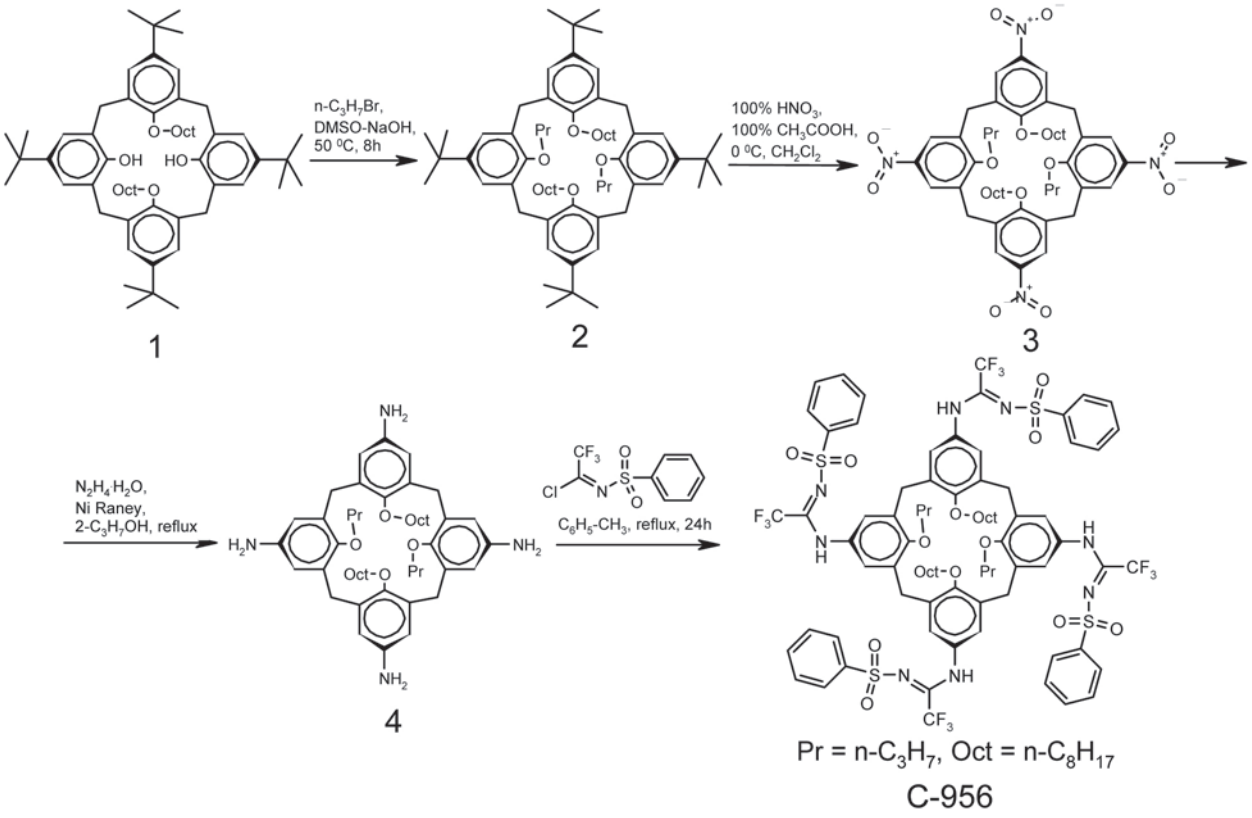
Synthesis

Calixarenes C-90 [13], C-716 [13], C-772 [12], C-715 [12] were synthesized accordingly to previously reported procedures.

Amphiphilic tetrasulfonylamidinocalixarene C-956 was synthesized accordingly Scheme 1 *via* four-step reactions pathway. Alkylation of dioctyloxy-*tert*-butylcalixarene **1** [14] with propylbromide in dimethylsulfoxide (DMSO) NaOH media afforded the tetraalkylderivative **2**. Subsequent *ipso*-nitration with nitric acid – glacial acetic acid mixture of this calixarene gave tetranitro compound **3**. Reduction of latter's nitro groups with hydrazine hydrate in presence of Raney Nickel resulted in tetraaminocalixarene **4**. Final stage is electrophilic substitution at carbon atom of N-sulfonylimidoylchloride which yielded, after two-stage purification procedure a desired tetrasulfonylamidine C-956 (Scheme 1).

*5,11,17,23-tetra-tert-butyl-25,27-dioctyloxy-26,28-dipropoxycalix[4]arene, **2***

Sodium hydroxide (50% water solution, 1.2 ml, 18 mmol NaOH) and then dioctylcalixarene **1** [15] (3 g, 3.43 mmol) were added to DMSO (35 ml). The reaction mixture was stirred for 15 min. 1-Brompropane (7.38 g, 6 mmol) was added by three equal portions every 30 min and the mixture was stirred



Scheme 1

for 8 h at 50-60°C. After cooling to 20°C the diluted hydrochloric acid was poured into the reaction mixture. Compounds **1** was extracted by chloroform (2×20 ml), organic layers were combined, washed with water (2×40 ml) and brine (40 ml) when dried with sodium sulfate overnight. Chloroform was evaporated the residue was washed with methanol. The title compound was obtained as pale-yellow solid, m 2.73 g, yield 82.8%. ^{1}H NMR ($CDCl_3$, 299.94 MHz), δ ppm: 6.79 and 6.77 two s (8H, ArH), 4.40 d (*J* = 12.7 Hz, 4H, $ArCH_{ax}Ar$), 3.83 and 3.82 two t (*J* = 7.3 Hz, 8H, O-C$\underline{H}_2$-CH_2-CH_3, O-C$\underline{H}_2$-CH_2-$(CH_2)_5$-CH_3), 3.13 d (*J* = 12.7 Hz, 4H, $ArCH_{eq}Ar$), 2.03 brm (8H, O-CH_2-C$\underline{H}_2$-CH_3, O-CH_2-C$\underline{H}_2$-$(CH_2)_5$-CH_3), 1.32-1.37 m, (20H, O-CH_2-CH_2-(C$\underline{H}_2$)$_5$-CH_3) 1.09 and 1.08 two s (36H, *t*-Bu), 1.00 t (*J* = 7.7 Hz, 6H, O-CH_2-CH_2-C$\underline{H}_3$) 0.91 brt (6H, O-CH_2-CH_2-$(CH_2)_5$-C$\underline{H}_3$).

*5,11,17,23-tetranitro-25,27-dioctyloxy-26,28-dipropoxycalix[4]arene, **3***

To ice-bath cooled solution of dioctyloxydipropoxycalixarene **2** (2.72 g, 2.84 mmol) in dry dichloromethane (DCM, 30 ml) was added dropwise mixture of 100% nitric acid (8 ml) and glacial acetic acid (26 ml). Reaction solution color was changed to blue-black, and then it discolored. After 6 h reaction mixture was poured into water (150 ml) and organic layer was separated. Water layer was washed with 20 ml of DCM, organic layers were combined, washed with diluted hydrochloric acid (50 ml), water (2×50 ml) and brine (50 ml) when dried with sodium sulfate overnight. DCM was evaporated the bright-yellow residue was dissolved in chloroform (15 ml) and precipitated by four portions of methanol (4×15 ml) upon heating. After cooling, the title compound was filtered and dried at air. Tetranitrocalixarene 3 was obtained as pale-yellow fine crystals, m 1.55 g, yield 60%. ^{1}H NMR ($CDCl_3$, 299.94 MHz), δ ppm: 7.59 and 7.56 two s (8H, ArH), 4.49 d (*J* = 14.1 Hz, 4H, $ArCH_{ax}Ar$), 3.99 and 3.94 two t (*J* = 7.5 Hz, 8H, O-C$\underline{H}_2$-CH_2-CH_3, O-C$\underline{H}_2$-CH_2-$(CH_2)_5$-CH_3), 3.42 d (*J* = 14.1 Hz, 4H, $ArCH_{eq}Ar$), 1.90 brm (8H, O-CH_2-C$\underline{H}_2$-CH_3, O-CH_2-C$\underline{H}_2$-$(CH_2)_5$-CH_3), 1.29-1.36 m (20H, O-CH_2-CH_2-(C$\underline{H}_2$)5-CH_3) 1.02 t (*J* = 7.3 Hz, 6H, O-CH_2-CH_2-C$\underline{H}_3$), 0.89 t (*J* = 7.1 Hz, 6H, O-CH_2-CH_2-$(CH_2)_5$-C$\underline{H}_3$).

5,11,17,23-tetramino-25,27-dioctyloxy-26,28-dipropoxycalix[4]arene, ***4***

A suspension of tetranitrocalixarene 3 (1.53 g, 1.68 mmol) and hydrazine hydrate (4 ml) in 2-propanol (125 ml) was heated up to 60°C. A 10% (weight) suspension of Raney Ni was added to calixarene suspension under stirring. Reaction mixture was refluxed 10 h, then filtered through zeolites and evaporated (15 mmHg, 70°C). Solid residue was twice re-evaporated from toluene (2×30 ml) and dried at vacuum (15 mmHg, 80°C). The title compound was obtained as pale-brown fine crystals, m 1.04 g, and yield 78%. ^{1}H NMR ($CDCl_3$, 299.94 MHz), δ ppm: 6.08 and 6.04 two s (8H, ArH), 4.29 d (J = 13.3 Hz, 4H, $ArCH_{ax}Ar$), 3.76 and 3.73 two t (J = 7.2 Hz, 8H, O-C$\underline{H}_2$-CH_2-CH_3, O-C$\underline{H}_2$-CH_2-$(CH_2)_5$-CH_3), 3.07 brs (8H, NH_2), 2.94 d (J = 13.3 Hz, 4H, $ArCH_{eq}Ar$), 1.86 m (8H, O-CH_2-C$\underline{H}_2$-CH_3, O-CH_2-C$\underline{H}_2$-$(CH_2)_5$-CH_3), 1.29-1.34 brm (20H, O-CH_2-CH_2-(C$\underline{H}_2)_5$-CH_3), 0.95 t (J = 7.4 Hz, 6H, O-CH_2-CH_2-C$\underline{H}_3$) 0.89 t (J = 7.0 Hz, 6H, O-CH_2-CH_2-$(CH_2)_5$-C$\underline{H}_3$).

5,11,17,23-tetrakis-triluoromethyl(phenylsulfonylimino)methylamino-25,27-dioctyloxy-26,28-dipropoxycalix[4]arene, ***C-956***

To a solution of N-(phenyl)sulfonylimidoylchloride [16] (1.16 g, 4.27 mmol) in dry toluene (15 ml) a solution of aminocalixarene 4 (0.79 g, 1 mmol) was added dropwise. Resulted mixture was refluxed for 24 h, then cooled and evaporated (15 mmHg, 70°C). Residue brown solid foam (1.58 g) was separated by column chromatography (silica gel 63-100 μM, Merck) using chloroform-methanol eluent with variable polarity (from 130:1 to 90:1). The obtained pale-brown solid (0.84 g) was recrystallized from 7 ml 2-propanol. The title compound was filtered as pale-yellow-brown crystals which were dried at air, m 0.7 g, yield 40.4%. ^{1}H NMR ($CDCl_3$, 299.94 MHz), δ ppm: 9-9.05 brs, (4H, NH), 7.91 d, (J = 6.4 Hz, 8H, *orto*-SO_2ArH), 7.49 m (12H, *meta*- and *para*-SO_2ArH), 6.57 brs (8H, ArH), 4.37 d (J = 12.9 Hz, 4H, $ArCH_{ax}Ar$), 3.87 brm (8H, O-C$\underline{H}_2$-CH_2-CH_3, O-C$\underline{H}_2$-CH_2-$(CH_2)_5$-CH_3), 3.06 d (J = 12.9 Hz, 4H, $ArCH_{eq}Ar$), 1.93 m (8H, O-CH_2-C$\underline{H}_2$-CH_3, O-CH_2-C$\underline{H}_2$-$(CH_2)_5$-CH_3), 1.31-1.37 brm (20H, O-CH_2-CH_2-(C$\underline{H}_2)_5$-CH_3), 1.01 t (J = 6.9 Hz, 6H, O-CH_2-CH_2-C$\underline{H}_3$) 0.90 t (J = 6.4 Hz, 6H, O-CH_2-CH_2-$(CH_2)_5$-C$\underline{H}_3$). ^{19}F NMR ($CDCl_3$, 188.14 MHz), δ, ppm: -65.7 s (6F), -66.0 s (6F). ^{13}C ($CDCl_3$, 125.73 MHz), δ ppm: 154.53, 153.49, 138.85, 135.67, 132.89, 132.75, 129.06, 128.99, 128.87, 128.78, 126.68, 126.62, 126.37, 77.20, 31.89, 31.84, 30.70, 30.06, 29.67, 29.40, 26.10, 23.19, 22.63, 14.10, 14.05.

Synthesis of three-substituted calixarene C-957 (Scheme 2) started from trinitro-dipropoxycalixarene 7, obtained through careful excessive ipso-nitration of tert-butyl-dipropoxycalixarene with mixture of 75% nitric acid and glacial acetic acid. Reduction of notrocalixarene 7 with hydrazine hydrate in presence of Raney Nickel gave the trisaminocalixarene 8. Final reaction with N-sulfonylimidoylchloride afforded a desired trisulfonylamidine C-957.

Ipso-nitration of tert-butyl-2,4-dipropoxycalix[4]arene ***1***

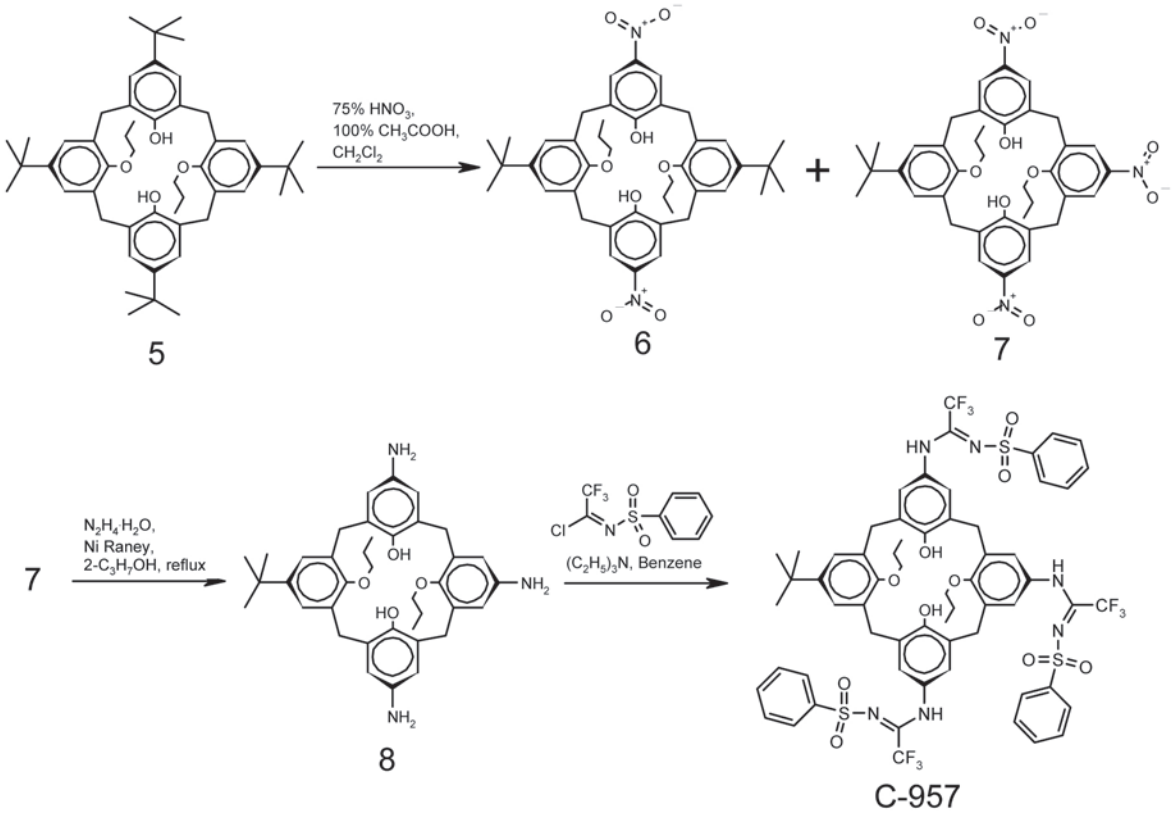
To solution of 5,17,11,23-tetra-*tert*-butyl-26,28-dihydroxy-25,27-dipropoxycalix[4]erene **5** [17] (0.49 g, 0.67 mmol) in DCM (18 ml) a mixture of 75% nitric acid (2 ml) and glacial acetic acid (2 ml) was added dropwise at 25°C under stirring. Obtained solution becomes deep dark purple color, which rapidly changed to orange-yellow. Reaction mixture was stirred for 20 min and poured into water (100 ml). Organic layer was separated, washed with water and brine (by 20 ml) and leaved over sodium sulphate for 12 h. Solvent was removed on rotor evaporator, residue, orange solid, was separated by column chromatography (eluent: chloroform, R_f(**7**) 0.15, R_f(**6**) 0.5).

5,11,17-trinitro-23-tert-butyl-26,28-dihydroxy-25,27-dipropoxycalix[4]arene ***7***

Yellow crystalline compound, yield 25.5%. m.p. = 280–281°C. ^{1}H NMR ($CDCl_3$, 299.94 MHz), δ, ppm: 9.05 s (2H, O$\underline{H}$), 8.11 s (4H, O_2N-Ar$\underline{H}$), 7.83 s (2H, O_2N-Ar$\underline{H}$), 6.95 s (2H, *t*-Bu-Ar$\underline{H}$), 4.36 and 4.21 two d (J = 13.4 Hz, 4H, $ArC\underline{H}_{ax}Ar$), 4.06 and 4.01 two d (J = 6.2 Hz, 4H, O-C$\underline{H}_2$-CH_2-CH_3), 3.62 and 3.55 two d (J = 13.4 Hz, 4H, $ArC\underline{H}_{eq}Ar$), 2.09 m (4H, O-CH_2-C$\underline{H}_2$-CH_3), 1.32 and 1.29 two t (J = 7.5 Hz, 6H, O-CH_2-CH_2-C$\underline{H}_3$), 1.07 s (9H, *t*-Bu). Found, %: C 64.79; H 5.99; N 5.64. $C_{38}H_{41}N_3O_{10}$. Calculated, %: C 65.23; H 5.91; N 6.00.

5,11,17-trisamino-23-tert-buty-26,28-dihydroxy-25,27-dipropoxycalix[4]arene ***8***

The suspension of trinitrocalixarene 7 (2 g, 2.86 mmol) and hydrazine hydrate (3 ml) in 2-propanole (120 ml) was heated to 70 ºC and water suspension of Raney nickel (10% by weight, 0.3 ml) was added to obtained suspension. Reaction mixture was refluxed under stirring for 7 h till full dissolution of calixarene and discoloration. The reaction mixture was cooled and Raney nickel was filtered off through silica gel (1–1.5 cm layer). Filtrate was evaporated by rotor evaporator and re-evaportated from toluene (20 ml). Residue was crystallized from



Scheme 2

toluene and dried in vacuum (0.1 mmHg, 70ºC, 2 h). The trisaminocalixarene **8** was obtained as pale-yellow crystals that slow turned pink at air. Yield 1.7 g, 97%. m.p. = 250–252°C. ^{1}H NMR ($CDCl_3$, 299.94 MHz), δ, ppm: 7.90 brs (2H, OH), 6.93 s (2H, t-Bu-ArH), 6.5 d (*J* = 4.0 Hz, 4H, NH_2-ArH), 6.11 s (2H, NH_2-ArH), 4.25 and 4.19 two d (*J* = 12.8 Hz, 2H, ArCH_{ax}Ar), 3.91 and 3.85 two d (*J* = 6.4 Hz, 4H, O-CH_2-CH_2-CH_3), 3.12 and 3.25 two d (*J* = 12.7 Hz, 4H, ArCH_{eq}Ar), 3.40–3.00 brs (6H, NH_2), 2.03 m (4H, O-CH_2-CH_2-CH_3), 1.23 two t (*J* = 7.2 Hz, 6H, O-CH_2-CH_2-CH_3), 1.12 s (9H, t-Bu).

5,11,7-tri(phenylsulphonylimino)trifluoro-methyl-methylamino-23-tert-butyl-26,28-dihydroxy-25,27-dipropoxycalix[4]arene ***C-957***

A solution of imidoylchloride [16] (0.35 g, 1.29 mmol) in benzene (10 ml) was added to a solution of trisaminocalix[4]arene **8** (0.25 g, 0.41 mmol) and triethylamine (0.12 g, 1.19 mmol) in benzene (30 ml). Reaction mixture was stirred for 20 h. Precipitate formed was filtered and washed by benzene and twice by water-methanol mixture (9:1, v/v, 20 ml). Residue precipitate, sulfonylamidine calixarene **C-957** was dried on air. Colorless crystal compound: yield 0.42 g, 78%; m.p. = 225–227ºC. ^{1}H NMR ($(CD_3)_2S$=O, 299.94 MHz, 328 K), δ, ppm: 10.5 brs (3H, NH), 8.31 s (2H, OH), 7.86 two d (*J* = 8.3 Hz, 4H, SO_2-PhH *orto*), 7.78 d (*J* = 8.3 Hz, 2H, SO_2-PhH *orto*), 7.55 m (9H, SO_2-PhH), 7.29 and 7.13 two d (*J* = 2.5 Hz, 4H, N-ArH), 6.92 s (2H, t-Bu-ArH), 6.84 s (2H, N-ArH), 4.18 and 4.14 two d (*J* = 13.3-5 Hz, 4H, ArCH_{ax}Ar), 3.99 and 3.96 two t (*J* = 6.1-3 Hz, 4H, O-CH_2-CH_2-CH_3), 3.36 and 3.23 two d (*J* = 13.3-5 Hz, 4H, ArCH_{eq}Ar), 1.98 m (4H, O-CH_2-CH_2-CH_3), 1.25 and 1.24 two t (*J* = 7.4 Hz 6H, O-CH_2-CH_2-CH_3), 1.04 s (9H, *t*-Bu). ^{19}F NMR ($(CD_3)_2S$=O, 188.14 MHz), δ, ppm: -65.6 s (6F), -66.1 s (3F). ^{13}C ($CDCl_3$, 125.73 MHz), δ ppm: 149.49, 149.24, 148.84, 143.64, 141.84, 141.43, 137.33, 133.95, 132.61, 129.41, 129.29, 128.14, 127.72, 127.41, 127.31, 125.61, 120.26, 119.79, 78.15, 77.95, 33.95, 31.06, 30.86, 22.96, 21.05, 20.9, 10.82. Found, %: C, 56.60, H, 5.06, N, 6.33, S, 7.06. $C_{62}H_{59}F_9N_6O_{10}S_3$. Calculated, %: C, 56.61, H, 4.52, F, 13.00, N, 6.39, S, 7.31.

Synthesis of disulfonylamidinocalixarene C-960 was performed by one-step reaction of corresponding diaminocalixarene **9** [17] with N-sulfonylimidoylchloride. Desired compound is separated from reaction mixture by relatively easy procedure with reasonable yield.

5,17-bis-trifluoromethyl(phenylsulfonylimino) methylamino-25,27-dipropoxy-26,28-dihydroxycalix[4]arene ***C-960***

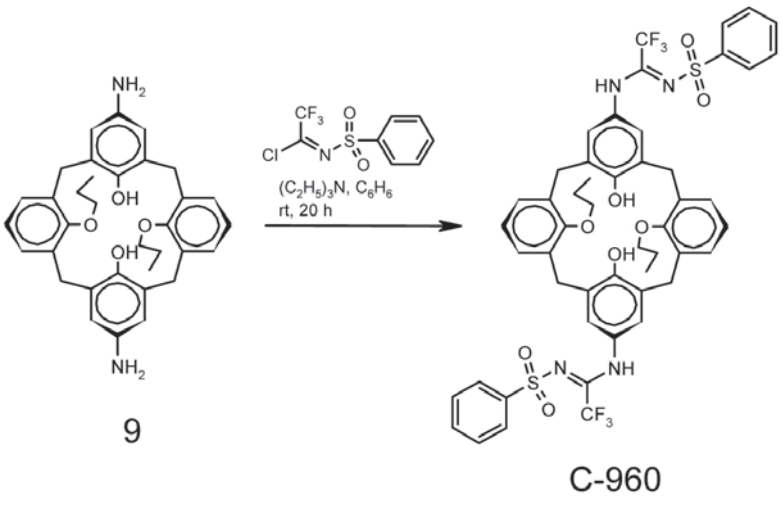
A solution of aminocalixarene **9** [18] (0.54 g, 1 mmol) and triethylamine (0.2 g, 2 mmol) in hot dry benzene (60 ml) was added dropwise to a stirred solution of the imidoyl chloride [16] (0.61 g, 2.2 mmol) in 10 ml of benzene. The reaction mixture was stirred at room temperature for 14 h. Precipitate was filtered, washed with benzene and water-methanol mixture (2×15ml, 9:1, v/v). Residue was dried at air. The title compound, bis-sulfonylamidinecalixarene **C-960** was obtained as pale-rose fine crystals, m 0.61 g, yield 61.5%. ^{1}H NMR ($CDCl_3$, 299.94 MHz), δ ppm: 9.73 s (2H, NH), 8.70 s (2H, OH), 7.76 brd (4H, *orto*-SO_2ArH), 7.23 m (6H, *meta*- and *para*-SO_2ArH), 7.03 s (4H, ArH), 6.80 d (*J* = 7.2 Hz, *meta*-Ar<u>H</u>), 6.59 t (*J* = 7.2 Hz, *para*-Ar<u>H</u>), 4.27 d (*J* = 12.9 Hz, 4H, $ArCH_{ax}Ar$), 3.98 t (*J* = 5.9 Hz, 4H, O-C<u>H</u>$_2$-CH_2-CH_3), 3.39 d (*J* = 12.9 Hz, 4H, $ArCH_{eq}Ar$), 2.06 m (4H, O-CH_2-C<u>H</u>$_2$-CH_3), 1.33 t (*J* = 7.4 Hz, 6H, O-CH_2-CH_2-C<u>H</u>$_3$. ^{19}F NMR ($(CD_3)_2S$=O, 188.14 MHz), δ, ppm: -65.6 s. ^{13}C ($CDCl_3$, 125.73 MHz), δ ppm: 151.55, 139.95, 132.75, 132.66, 132.08, 129.02, 128.93, 128.44, 128.35, 128.12, 127.20, 127.16, 126.13, 125.18, 78.30, 31.04, 23.27, 10.71.

Tetrasulfonylamidinocalixarene C-975 was synthesized *via* reaction of tetrakisaminocalixarene [19] and N-sulfonylimidoylchloride with lower molecular weight. Crude product was purified in two steps by column chromatography and further precipitation from methanol-water system.

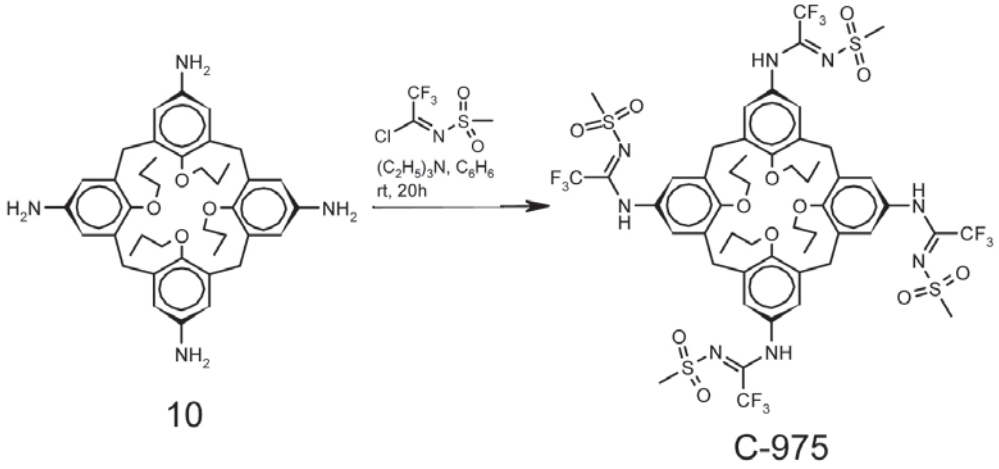
5,11,17,23-tetrakis-triluoromethyl(methylsulfonylimino)methylamino-25,26,27,28-tetrapropoxycalix[4]arene, ***C-975***

A solution of aminocalixarene **10** [18] (0.33 g, 0.5 mmol) and triethylamine (0.21 g, 2.06 mmol) in dry benzene (35 ml) was added dropwise to a stirred solution of the N-(methyl)sulfonylimidoylchloride [16] (0.46 g, 2.2 mmol) in 10 ml of dry benzene. Resulted mixture was stirred for 1h and then refluxed for 1 h, then cooled and filtrated. Filtrate was evaporated (15 mmHg, 70°C), residue brown solid foam was separated by column chromatography (silica gel 63-100 μm, Merck) using chloroform-methanol eluent (80:1). The obtained pale-brown solid (0.60 g) was dissolved in 8 ml of methanol and precipitated by 1.5 ml of water. The title compound was filtered as pale-yellow crystals which were dried at air, m 0.27 g, yield 36.4%. ^{1}H NMR ($CDCl_3$, 299.94 MHz), δ ppm: 8.92 s (4H, NH), 6.78 s (8H, ArH), 4.42 d (*J* = 13.1 Hz, 4H, $ArCH_{ax}Ar$), 3.87 t (*J* = 7.5 Hz, 8H, O-C<u>H</u>$_2$-CH_2-CH_3), 3.20 d (*J* = 13.1 Hz, 4H, $ArCH_{eq}Ar$), 3.09 s (12H, C<u>H</u>$_3$$SO_2$), 1.97 m (8H, O-$CH_2$-C<u>H</u>$_2$-$CH_3$), 1.01 t (*J* = 7.2 Hz, 6H, O-CH_2-CH_2-C<u>H</u>$_3$). ^{19}F NMR ($CDCl_3$, 376.50 MHz), δ, ppm: -66.72 s. ^{13}C ($CDCl_3$, 125.73 MHz), δ ppm: 156.12, 135.10, 129.05, 126.12, 118.33, 115.51, 77.20, 42.83, 30.73, 23.17, 10.18.

Animals. All experiments were carried out in accordance with European Community Council Directive 2010/63/EU of 22 September 2010. Experimental protocols were approved by the Animal Care and Use Committee of the Palladin Institute of



Scheme 3



Scheme 4

Biochemistry of National Academy of Sciences of Ukraine (the Protocol from 19/05/2021).

Wistar female rats, 100-120 g body weight, were obtained from the vivarium of Strazhesko Institute of Cardiology, National Academy of Medical Sciences of Ukraine. Animals were kept at animal facility of Palladin Institute of Biochemistry of National Academy of Sciences of Ukraine. Animals were housed in a quiet and temperature-controlled room (22-23°C) and provided with water and dry food pellets ad libitum. Before removing the uterus, rats were anesthetized with isoflurane and decapitated. The total number of rats scarified in this study was 12.

Porcine uteri of adult animals were obtained from the local abattoirs. Uteri containing endometrial and myometrial tissues were cut off and frizzed in liquid nitrogen. The total number of pigs used in this study was 5.

Isolation of PM fraction. Isolation of PM fraction of porcine myometrium cells was made by density gradient centrifugation, which is the conventional way to obtain subcellular fractions and was described in [20, 21]. Briefly, a homogenate of cells is ultracentrifugated at 100,000 g in discontinuous gradient of sucrose density. PM vesicles are harvested in 15% sucrose solution at the border with 30% sucrose solution. The protein content in the membrane fraction was determined by the Bradford method [22].

Isolation of SMCs. SMCs were isolated from a uterus of non-pregnant rats using a modification of the previously described method [23]. The rats were estrogenized one day before the experiment. After separation of fat and connective tissue, the uterus was placed in warm (36°C) Hanks medium (solution A) of the following composition (mM): 136.9 NaCl, 5.36 KCl, 0.44 KH_2PO_4, 0.26 $NaHCO_3$, 0.26Na_2HPO_4, 1.26 $CaCl_2$, 0.4 $MgCl_2$, 0.4 $MgSO_4$, 5.5 glucose, 10 Hepes-Tris buffer (pH 7.4). The tissue was cut into pieces (~2×2 mm) and washed to free of blood and calcium ions (3 times for 5 min) using solution B (solution A containing 0.03 mM $CaCl_2$ instead of $MgCl_2$ and $MgSO_4$). The pieces were incubated for 20 min at 36ºC in dissociation medium with constant mixing. The medium contained 0.1% collagenase, 0.1% bovine serum albumin and 0.01% soybean trypsin inhibitor in solution B. To accelerate cell dissociation the tissue preparation was pipetted for 1-2 min using a glass pipette. Solution B containing dissociated cells was decanted and the remaining tissue preparation was placed into a new portion of dissociated medium. The whole procedure was repeated 5-6 times. The two first portions of solution B containing dissociated cells were discarded because they contained tissue fragments and damaged cells. The last three or four portions were pooled and centrifuged for 10 min at 80 g. The sedimented cells were washed with solution containing 25 mM Hepes-Tris buffer (pH 7.4), 150 mM NaCl, 0.5% bovine serum albumin and centrifuged again. Then the cells were suspended in solution containing 25 mM Hepes-Tris buffer (pH 7.4), 150 mM NaCl and kept at 4°C. Obtained suspension contained (5-7)×10^6 myocytes per 1 ml. The amount of viable cells determined with Trypan Blue staining was 75-90%. Cell amount was counted using a hemocytometer.

Enzymatic assay. The PMCA (EC 7.2.2.10) activity was determined in the PM fraction of the myometrium cells at 37°C in 0.4 ml of medium containing 3 mM ATP, 3 mM $MgCl_2$, 0.95 mM $CaCl_2$, 25 mM NaCl, 125 mM KCl, 1 mM EGTA, 20 mM Hepes-Tris buffer (pH 7.4), 1 mM NaN_3 (an inhibitor of mitochondrial ATPase [24]), 1 mM ouabain (an inhibitor of Na^+,K^+-ATPase [25, 26]), 0.1 μM thapsigargin (a selective inhibitor of Ca^{2+},Mg^{2+}-ATPase of endo(sarco)plasmic reticulum [24]), and 0.1% digitonin (a factor of PM perforation [27]). The protein content of the membrane fraction in the sample was 20-30 μg. The incubation time was 5 min. The reaction was initiated by addition into the medium of an aliquot (50 μl) of the PM suspension and was stopped by addition to the reaction mixture of 1 ml of "stop" solution with the following composition: 1.5 M sodium acetate, 3.7% formaldehyde, 14% ethanol, 5% TCA (pH 4.3 at 8°C). The amount of the reaction product P_i was determined with method Rathbun and Betlach [28]. The PMCA activity was calculated by the difference between values of the ATPase activity in the absence and in the presence of calcium ions. The calculations done using program "MAXCHEL" shows that free Ca^{2+} concentration at described physicochemical conditions of incubation medium is 1 μM. In our experiments the mean value of the specific enzymatic activity of PMCA of the uterine myocyte PM was 3.4 ± 0.3 μmol P_i/mg protein per 1 h (n = 7).

The dependences the activity of PMCA on concentration of calixarenes were used to determine values of the inhibition coefficients IC_{50} and cooperativity coefficients n, which were calculated using the Hill equation: $A = A_0 \times IC_{50}{}^n/(IC_{50}{}^n + [I]^n)$, where A_0 and A are specific enzymatic activities in the absence and in the presence of calixarenes in the incubation medium in the concentration [I] respectively. Results of the studies were treated statistically with Student's t-test. Kinetic and statistical calculations were carried out using the MS Excel software.

Confocal microscopy. The measurements of calixarene induced increase of [Ca^{2+}] in SMCs were carried out by laser scanningconfocal microscopy using a Zeiss LSM 510 "META" microscope (Carl Zeiss, Zeiss LSM Image Browser Version 4.0.0241 Software). SMCs were attached to a glasssurface with poly-L-lysine. The confocal microscopy analysis of immobilized cells was carried out on elongated cells with the nuclei well painted by fluorescent probe Hoechst 33342 and loaded with 10 μM Ca^{2+}-sensitive probe fluo-4 AM for 20 min at a room temperature. The concentration of Ca^{2+} in the cytoplasm was measured in calixarene-free medium and following the addition of one of calixarene (20μM).

Dynamic light scattering. Effective hydrodynamic diameter (EHD) of SMCs was measured by dynamic light scattering (DLS) using a Zetasizer-3 instrument equipped with multi computing correlator type 7032 ce (Malvern Instruments, UK). The size distribution was determined by DLS at a room temperature. Mean size represents the average of 20 readings. Experiments were repeated five times independently. Scattered light was detected in an angle of 90°. The results were analyzed by PCS-Size mode v1.61 software.

Materials. Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich.

Results and Discussion

The effect of quantity and arrangement of phenylsulfonylamidine groups on the upper rim of calixarenes on PMCA and cytoplasmic Ca^{2+} concentration of SMCs

To determine a role of quantity and arrangement of phenylsulfonylamidine groups on the upper rim of the calixarene molecule for inhibition of PMCA we evaluated the influence on mentioned enzymatic activity of calixarenes C-716, C-772 and C-957 which have several structural distinctions compared to calixarene C-90 (Fig. 1).

Namely, calixarenes C-716 and C-772 have only two phenylsulfonylamidine groups in distal or proximal positions at the upper rim of its molecules and *tert*-butyl groups at the other para-positions of macrocycle phenolic rings. Calixarene C-957 has three phenylsulfonylamidine groups on the upper rim of calixarene macrocycle and *tert*-butyl group on forth phenol residue (Fig. 1). Although a structure of lower rim of the calixarenes C-716, C-772, C-957 and C-90 is different because of varied quantity and spatial location of propoxyg roups, previously we assumed that the modification of just upper rim of calixarene C-90 was crucial for inhibitory properties of the last one for PMCA [12]. To determine the effect of calixarenes C-957, C-772 and C-716 on PMCA we used them in the concentration range from 10^{-8} to 10^{-4} M and demonstrated that all calixarenes decreased mentioned activity with dose-dependent manner but with different efficiency (Fig. 2). The kinetic parameters, which reflect the efficiency of PMCA inhibition by corresponding calixarene, were calculated using shown dependences (Table).

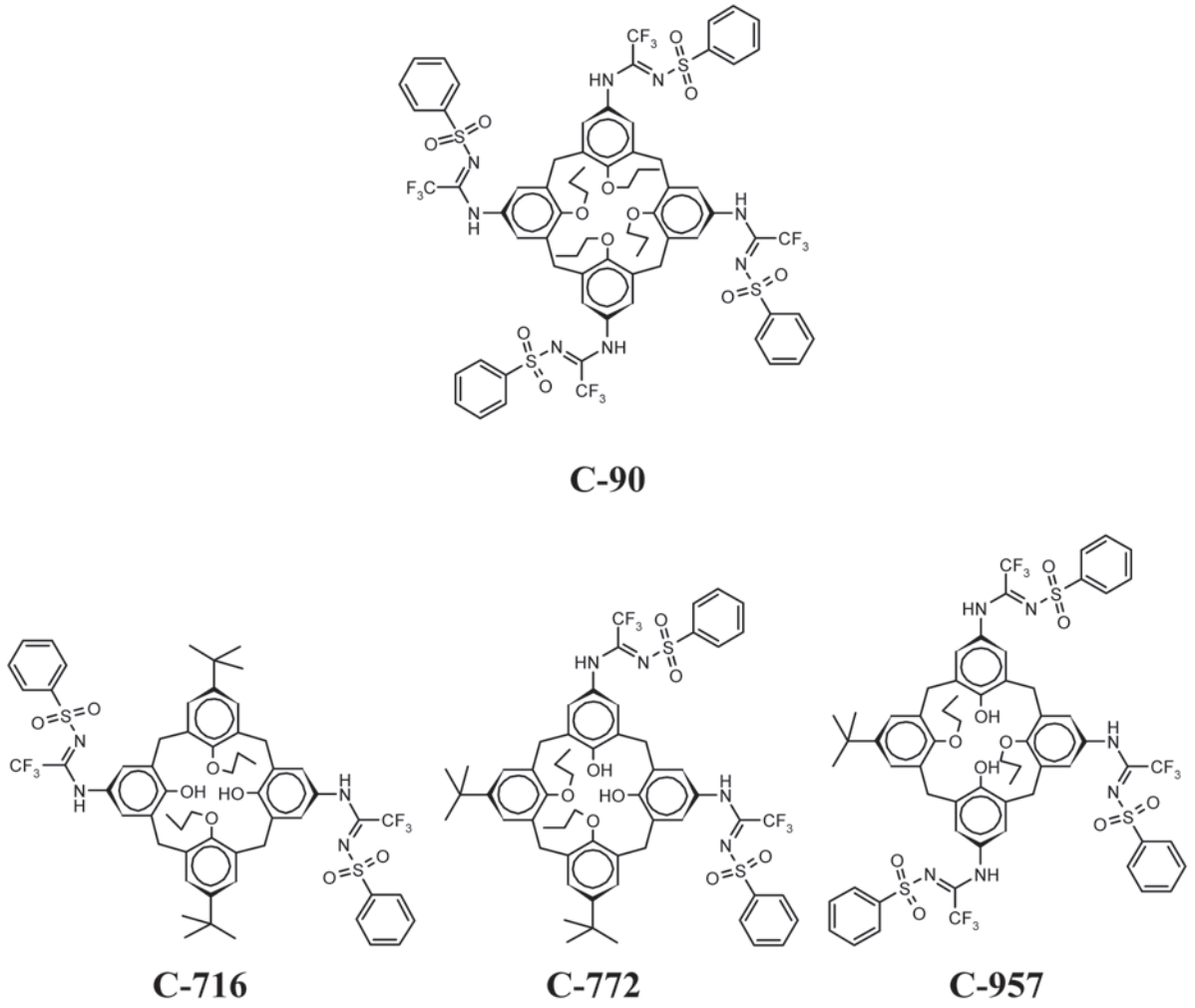


Fig. 1. Calixarene C-90 and its analogs C-716, C-772, C-957 which differ by quantity and arrangement of phenylsulfonylamidine groups on the upper rim

According to obtained results (Table), calixarene C-957 with three phenylsulfonylamidine groups has inhibition coefficient IC_{50} = 29.2 ± 0.9 that is higher than the same coefficient of calixarene C-90. Calixarenes C-716 and C-772 which have two phenylsulfonylamidine groups are even less effective inhibitors of PMCA. Their inhibition coefficients are higher than calixarene C-957 and equal 53.4 ± 3.6 and 165 ± 20 μM respectively. Thus, we make conclusion that increasing of quantity of sulfonylamidine groups at the upper rim of calixarene macrocycle led to rise of PMCA inhibition efficiency.

All kinetic calculations were made from PMCA activity curves shown at Figs. 2, 5 and 8 using Hill equation (see methods).

Moreover, comparison of the inhibitory efficiencies of calixarenes C-716 and C-772, which are spatial isomers, leads us to conclusion that distal arrangement of sulfonylamidine groups is more effective for PMCA inhibition (Fig. 2, Table) than proximal one. Probably the distance between phenylsulfonylamidine residues is also important parameter for supplying the complementarity of calixarene structure to the corresponding chemical groups on the surface of PMCA molecule.

Since PMCA plays a key role in the termination of the calcium signal in uterine myocytes, therefore, it is logical to assume that its inhibition may leads to an increase in the $[Ca^{2+}]$ in myocytes. In this context, the following important question arises: what is the PMCA inhibition efficiency of calixarenes is enough for significant change in $[Ca^{2+}]$? Therefore, the analysis of $[Ca^{2+}]$ changes, using confocal microscopy, under the action of similar calixarenes which differ

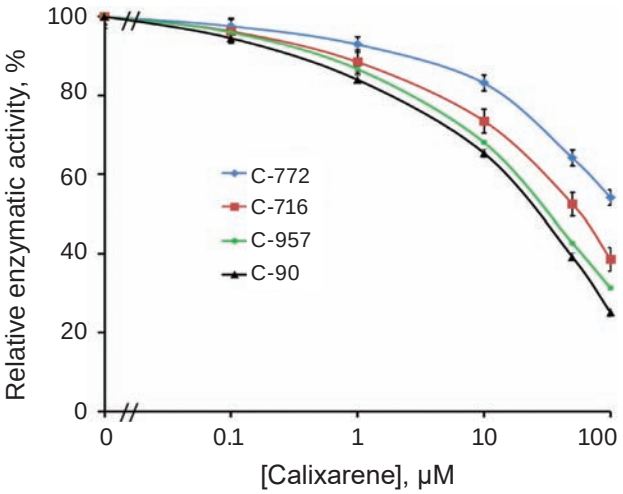


Fig. 2. The inhibition of PMCA activity of myometrium cells by calixarenes C-772, C-716, C-957 and C-90 (n = 5). 100% is values of enzymatic activity without calixarenes in incubation medium

Table. The kinetic parameters of myometrium PMCA activity inhibition by the calixarenes (n = 5)

Calixarene	Relative enzymatic activity at calixarene concentration 100 μM, %	Inhibition coefficient IC_{50}, μM	Hill coefficient *n*
C-90	25.0 ± 0.3	20.2 ± 0.5	0.55 ± 0.02
C-957	31.2 ± 0.6	29.2 ± 0.9	0.57 ± 0.02
C-716	38.5 ± 1.9	53.4 ± 3.6	0.52 ± 0.02
C-772	54.1 ± 0.6	165 ± 20	0.51 ± 0.04
C-715	79.3 ± 0.9	–	–
C-960	40.3 ± 0.5	50.0 ± 0.5	0.54 ± 0.01
C-975	29.7 ± 0.9	26.8 ± 0.9	0.57 ± 0.02
C-956	20.8 ± 0.4	15.0 ± 0.5	0.55 ± 0.01

by the structure of substituents, can give us the answer to the question.

It was shown that the addition of 20 μM calixarene C-90 to immobilized myocytes resulted in a sharp increase of fluo-4 fluorescence that corresponded to an increase of $[Ca^{2+}]$ in the cell (Fig. 3). It should be noted that the fluorescence level of background and fluorescence dye Hoechst, which was localized mainly in the nucleus of SMCs, was not changed by calixarene C-90 or any other calixarene. For 2.5 min, the $[Ca^{2+}]$ was lowered and set at a same level than before the introduction of C-90, indicating the involvement of compensatory systems for the extraction of Ca^{2+} out the cell. In the case of decreased activity of PMCA, an adaptive activation of sarco/endoplasmatic reticulum Ca^{2+},Mg^{2+}-ATPase, Na^+/Ca^{2+} exchanger and mitochondrial Ca^{2+} uniporter, which removing Ca^{2+} from the cytosol, is possible, because namely mentioned systems have lower affinity to Ca^{2+} and can be activated by its high $[Ca^{2+}]$, for example, during contraction of SMCs [3].

The same experiments were also carried out with other calixarenes in the same conditions except three or two time consequent addition of each calixarene. First injection of calixarene C-957 induced very similar responses of probe fluorescence (Fig. 3). Although efficiency of PMCA inhibition by this calixarene is lower than efficiency of calixarene C-90, the increase of $[Ca^{2+}]$ was even higher and sharper. Such effect can be rationalized by higher solubility or penetration rate to SMCs, etc. of calixarene C-957, that we didn't check. Second and third addition of calixarene C-957 induced much lower effect on $[Ca^{2+}]$, probably because part of PMCA was still inhibited after first calixarene addition.

Calixarenes C-716 and C-772 which have only two phenylsulfonylamidine groups didn't show visible effects on $[Ca^{2+}]$ even after three time addition to SMCs. Perhaps these calixarenes can interact with other targets inside or outside cells that compete or prevent interaction with PMCA in whole cells. Since we determined PMCA activity in isolated PM fraction, absence of such probable targets after PM isolation can be explanation of observed results. Anyway, we can make a conclusion that calixarenes which inhibited PMCA caused stimulation of $[Ca^{2+}]$ growth in SMCs.

The effect of *tert*-butyl and phenylsulfonylimino groups on the upper rim of calixarenes on PMCA and cytoplasmic Ca^{2+} concentration of SMCs

Since the molecules of C-957, C-716 and C-772 in contrast to C-90 have also *tert*-butyl groups at the upper rim of calixarene macrocycle, it was necessary to check the assumption that namely the presence of *tert*-butyl groups in the structure of mentioned calixarenes was the reason of PMCA inhibition efficiency decrease. Therefore, it was appropriate to use *tert*-butyl depleted calixarene phenylsulfonylamidine C-960 to determine their effect on PMCA (Fig. 4).

Our results indicated (Fig. 5) that the influence of calixarenes C-716 and C-960 on the activity of

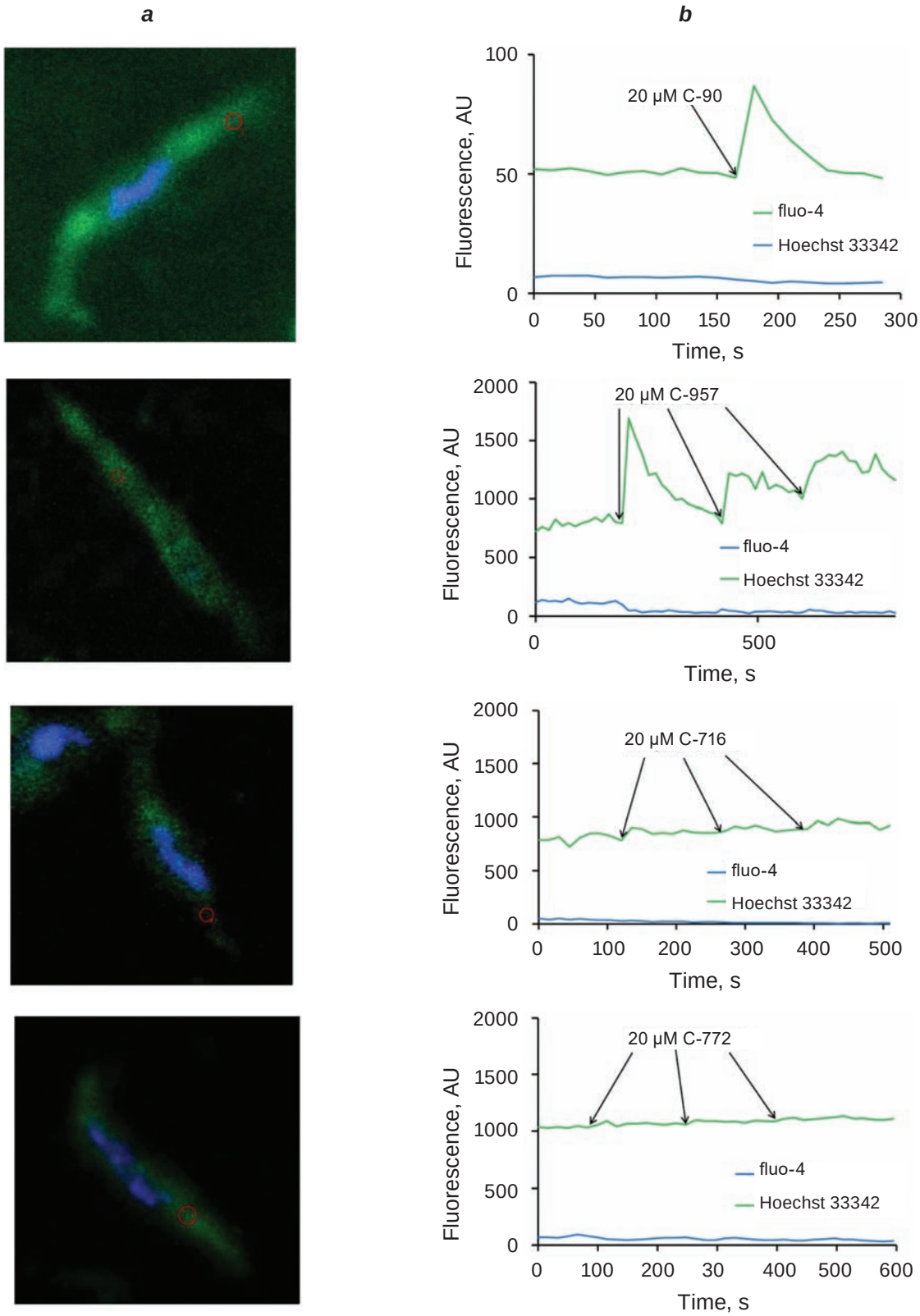


*Fig. 3. Typical effect of calixarenes C-90, C-957, C-716 and C-772 on the fluorescence of Ca^{2+} sensitive fluo-4 probe and DNA sensitive Hoechst 33342. (**a**) Photo of analyzed SMC: green is fluorescence of Ca^{2+} sensitive probe fluo-4, blue is fluorescence of DNA sensitive probe Hoechst 33342, red circle represents the region where fluorescence intensity was determined. (**b**) Time-dependent intensity of the probe fluorescence. Each calixarene was added at time marked by the arrows*

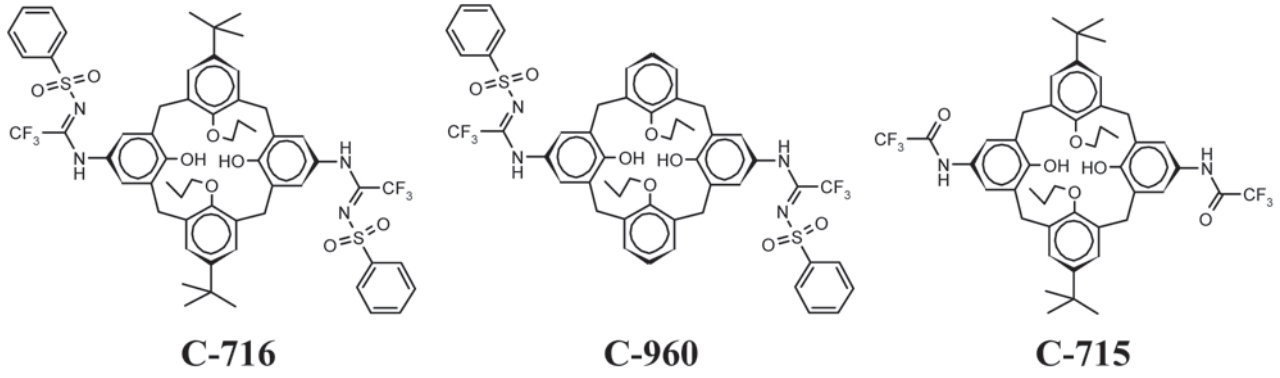


Fig. 4. Tert-butyl-calixarenesulfonylamididine C-716 and its tert-butyl depleted (C-960) and thifluoroacetamide (C-715) analogs

PMCA was virtually identical. Moreover, enzyme inhibiting coefficients (53.4 ± 3.6 and 50.0 ± 0.5 mM for calixarenes C-716 and C-960 respectively) are very similar (Table). So, it is obviously that the presence of *tert*-butyl residue doesn't effect on the efficiency of the PMCA inhibition.

We also checked the effect of calixarene C-960 on level of [Ca^{2+}] in SMCs and showed the slight rise of Ca^{2+}-sensitive probe fluorescence after first calixarene application and much higher increase of fluorescence after second adding (Fig. 6). Third calixarene injection didn't influence on [Ca^{2+}], perhaps, indicating effect of saturation after calixarene accumulation during first application. It is interesting that after each elevation of Ca^{2+} level we didn't observe returning of [Ca^{2+}] to the initial level, that can be the evidence of nonspecific inhibition all Ca^{2+}-pumping systems of SMCs. Comparing the effects of calixarenes C-960 and C-716 on the same parameter (Fig. 3 and 6), we can conclude also, that *tert*-butyl groups of calixarenes C-716 prevent the influence of this calixarene on [Ca^{2+}] in SMCs. As was assumed above, namely *tert*-butyl-containing calixarene structure might cause an affinity to other molecular targets in SMCs.

It is the most probable that namely phenylsulfonylamidine groups are crucial in enzyme inhibition. To make unambiguous conclusion, we used calixarene C-715, which had two trifluoroacetamidegroups at the upper rim of calixarenemacrocycle (Fig. 4). The dependence of the enzyme activity on calixarene concentration showed that the inhibition efficiency was very low and PMCA activity in the presence of maximal calixarene concentration 100 μM was only 79.3 ± 0.8% relative to control (Fig. 5, Table). Such low level of inhibition makes impossible to determine kinetic parameters, which we calculated for other calixarenes. That is why we also didn't check the effect of calixarene C-715 on [Ca^{2+}] in SMCs. However, we can unambiguously conclude that namely phenylsulfonylamidine groups of both calixarene C-716 and other calixarenescontaining mentioned residues are involved in enzyme inhibition molecular mechanism.

The effect of phenylgroups on the upper rim and octyl group on the lower rim of calixarenes on PMCA and cytoplasmic Ca^{2+} concentration of SMCs

The mechanism of PMCA inhibition by calixarene C-90 remains unknown and the site of interaction of calixarene with enzyme is not indentified.

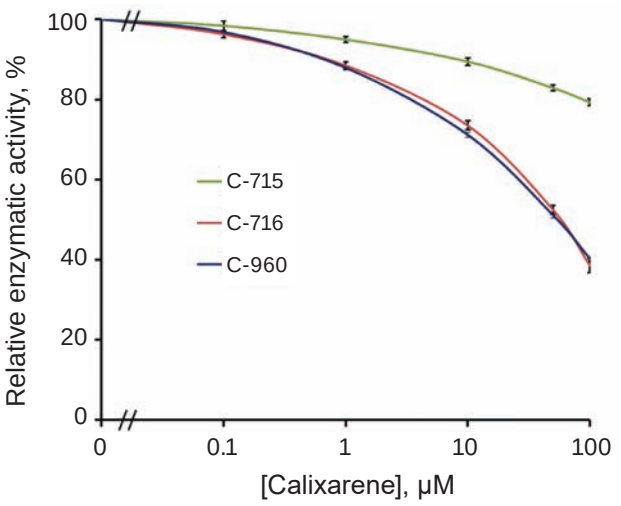


Fig. 5. The inhibition of PMCA activity of myometrium cells by calixarenes C-715, C-716, and C-960, (n = 5). 100 % is values of enzymatic activity without calixarenes in incubation medium

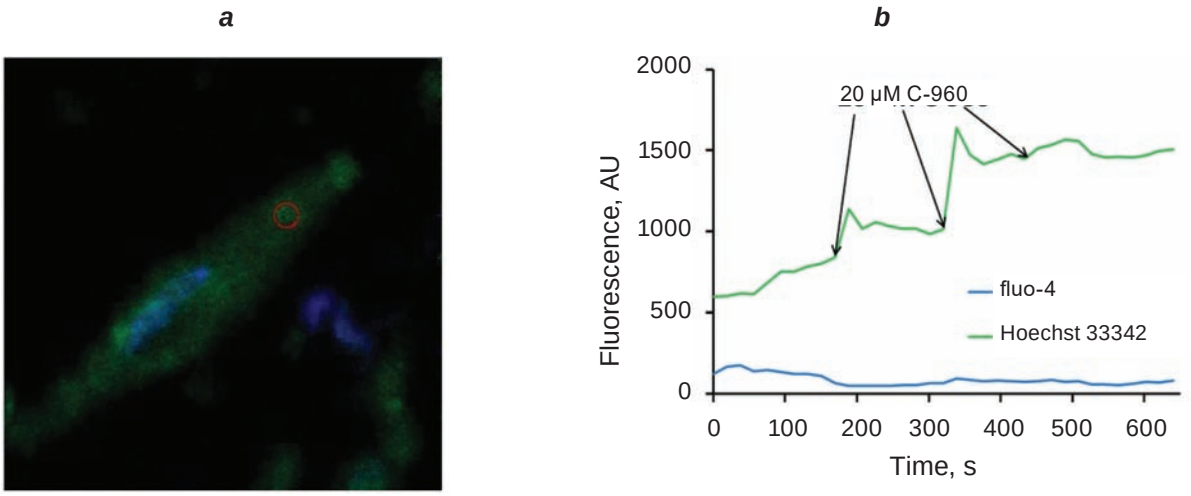


*Fig. 6. Typical effect of calixarene C-960 on the fluorescence of Ca^{2+} sensitive fluo-4 probes and DNA sensitive Hoechst 33342. (**a**) Photo of analyzed SMC: green is fluorescence of Ca^{2+} sensitive probe fluo-4, blue is fluorescence of DNA sensitive probe Hoechst 33342, red circle represents the region where fluorescence intensity was determined. (**b**) Time-dependentintensity of the fluorescence. Each calixarene was added at time marked by the arrows*

The question arises whether calixarenes interact with enzyme from aqueous environment or lipid environment of membrane and which of these mediums predominantly contains calixarenes. All mentioned calixarenes have predominantly hydrophobic properties, and are non-soluble in water that is probable disadvantage because water solubility is very important for using of compounds as inhibitors of enzymes or as pharmacological agent. Taking into consideration all mentioned above, calixarene C-90 analogs with the absent some hydrophobic groups have been researched (Fig. 7).

Thus, we synthesized calixarene N-methylsulfonylamidine C-975 which contained methyl residues instead of phenyl ones. Calixarene C-975 also inhibits PMCA activity (Fig. 8) and has inhibition coefficient IC_{50} = 26.8 ± 0.9 μM. So, the efficiency of inhibition of calixarene C-975 is slightly lower than the same of calixarene C-90 (Table). Obviously, phenyl residues of phenylsulfonylamidine groups don't participate in interaction with enzyme or such interaction is weak and doesn't cause any effect on enzyme function. So, we can conclude that namely sulfonylamidine fragments are crucial for inhibitory properties of calixarene C-90 and its analogs on activity of PMCA.

It is known that calixarenes can build in lipid membranes [6]. Change of hydrophobicity of calixarene molecules may influence on inhibition of PMCA, because such alteration has to lead to the change of calixarene molecule distribution between membrane and aqueous medium. Comparison of calixarenes with and without phenyl residues doesn't let us unambiguously conclude that namely hydrophobicity alters inhibitory efficiency of calixarenes since phenyl residues located in phenylsulfonylimino groups of calixarene upper rim may participate in interaction with complementary site of enzyme surface. That is why we used calixarene C-956, close analog of C-90, which has alteration in structure of lower rim. Previously, we have shown that the upper rim unsubstituted tetrapropoxycalixarene C-150 had no the inhibitory properties [12]. But the question remained opened whether the chemical groups located on the lower rim of the macrocycle influence on inhibition of PMCA. Calixarene C-956 has two more lipophilic octyl residues instead of propyl ones in calixarene C-90. Experimental data revealed that such modification of calixarene structure significantly didn't influence on inhibition of PMCA, however, slightly increased affinity of inhibitor to enzyme: inhibition coefficient IC_{50} was 15 ± 0.5 μM (Fig. 8, Table).

Thus, calixarene C-956 is the most effective inhibitor of PMCA among all investigated calixarenes. Obtained results evidence that calixarene hydrophobicity and therefore affinity to membrane environment has certain importance in interaction with enzyme. Inhibition of the solubilized PMCA by calixarene C-90 (IC_{50} = 58.5 ± 6.4 μM) endorsed

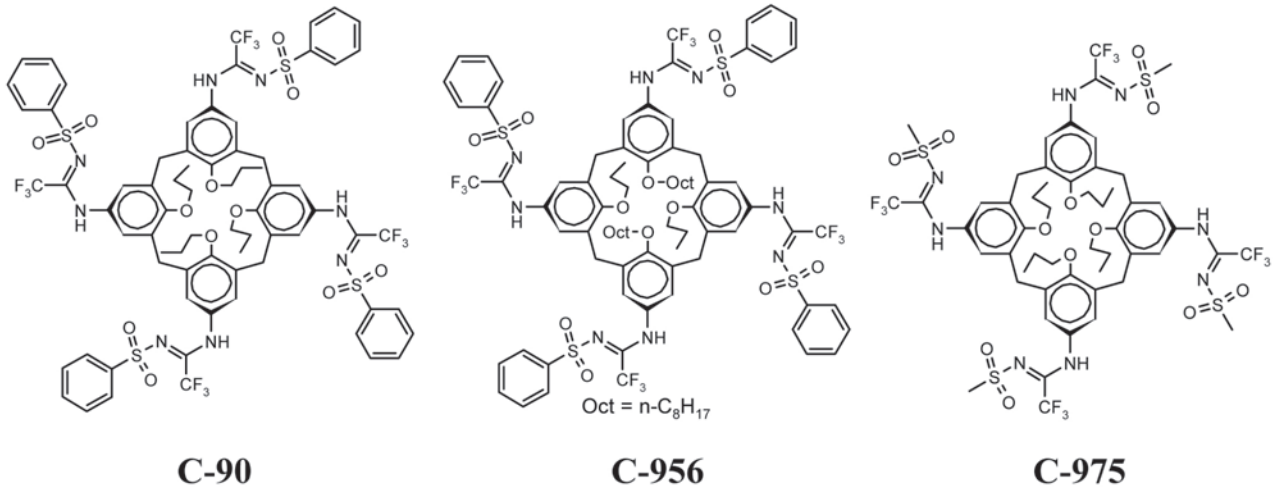


Fig. 7. Calixarene C-90 analogs, possessing methyl groups (C-975) on the upper rim and octyl group (C-956) on the lower rim of calixarene platform

this assumption about importance of membrane environment for inhibition of enzymatic activity of this transmembrane protein by calixarene [12]. So we make a suggestion that calixarenes inhibit PMCA from membrane lipid environment and therefore site of interaction with C-90 and other calixarenes locates on transmembrane domain of the protein molecule. Moreover, very close values of Hill coefficient for all calixarenes (Table) indicate that all investigated calixarenes effect on PMCA with the same mechanism.

Calixarenes C-975 and C-956 induced [Ca^{2+}] increase in SMCs similar to calixarene C-90 (Fig. 9). Decreasing of [Ca^{2+}] after sharp growth was also similar to effect of calixarene C-90, but this returning of [Ca^{2+}] didn't achieve initial level in all cases, that could be advantage in case of *in vivo* application of these calixarenes because such elevated prolonged Ca^{2+} level, probably, might cause also increase of basal muscle tone. The rise of [Ca^{2+}] after first addition of calixarene C-975 was not so high than after second calixarene addition and that indicated additional PMCA inhibition. In case of calixarene C-956, the biggest [Ca^{2+}] boost was after first calixarene injection and two consequent additions of calixarene induce the growth of [Ca^{2+}] only to the first maximal level of Ca^{2+}, probably, because of reaching of maximal PMCA inhibition even after the first calixarene application. Such difference in effects of two calixarenesis, certainly, caused by different efficiency of PMCA inhibition, since calixarene C-956 has almost twice less inhibition coefficient than calixcarene C-975 (Table).

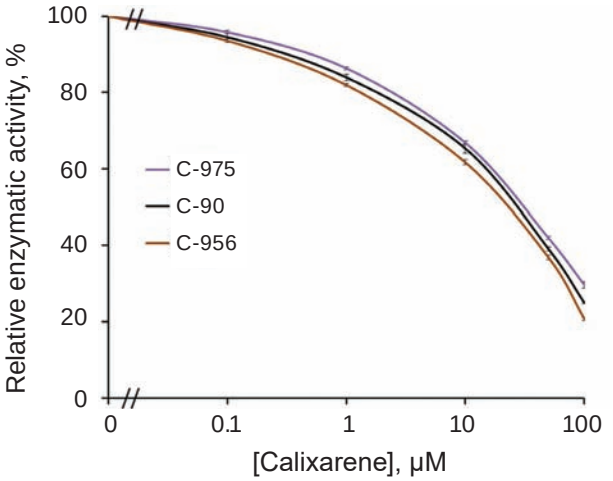


Fig. 8. The inhibition of PMCA activity of myometrium cells by calixarenes C-90 and more lipophilic C-956 and less lipophilic C-975 analogs with octyl and N-methylsulfonylamidine groups respectively (n = 5). 100 % is values of enzymatic activity without calixarenes in incubation medium

The effect of calixarenes on the effective hydrodynamic diameter of SMCs

Thus, enzymatic analysis and confocal microscopy have shown that effective inhibition of activity of PMCA by majority of calixarenes leads to an increase of intracellular [Ca^{2+}]. Such events can be assumed to change the contractile activity of the SMCs

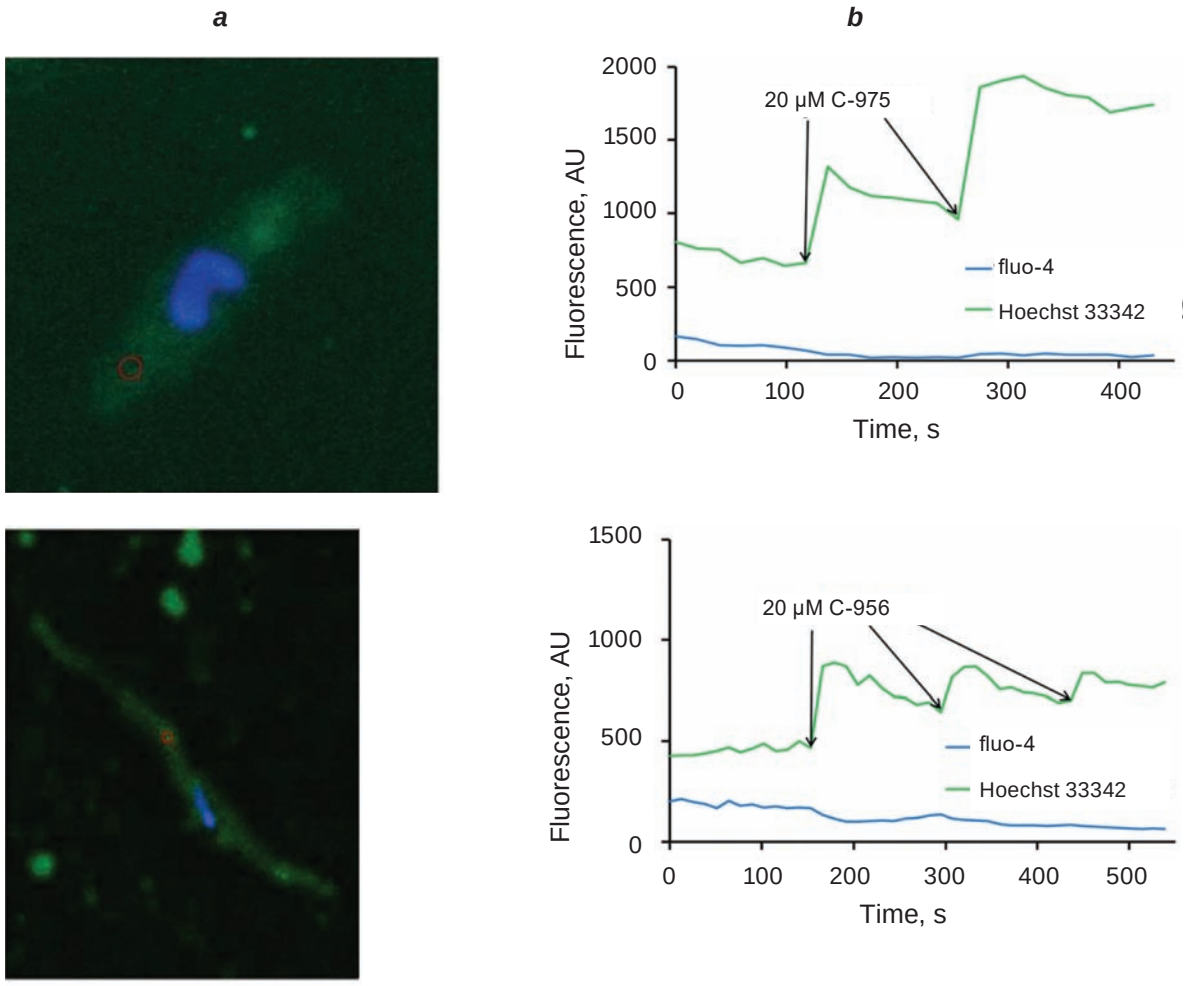


*Fig. 9. Typical effect of calixarenes C-975 and C-956 on the fluorescence of Ca^{2+} sensitive fluo-4 probes and DNA sensitive Hoechst 33342. (**a**) Photo of analyzed SMC: green is fluorescence of Ca^{2+} sensitive probe fluo-4, blue is fluorescence of DNA sensitive probe Hoechst 33342, red circle represents the region where fluorescence intensity was determined. (**b**) Time-dependent intensity of the probe fluorescence. Each calixarene was added at time marked by the arrows*

and affect their size, which can be detected by DLS method. According to the literature, compounds that increase the contractile activity of myocytes also decrease their EHD, which corresponds to the state of contraction of SMCs. DLS method showed that application to the SMC suspension 3 mM Ca^{2+}, 10 μM Ca^{2+}-ionophore A-23187, as well as the blockers of voltage dependent K^+-channels 1 mM 4-aminopyridine and 1 mM tetraethylammonium caused a decrease of EHD and an increase in the contractile activity of these cells [29]. That is why the next step of research was to test how calixarenes with different efficiency inhibited PMCA affect EHD of the SMCs by DLS method.

The average EHD of the SMCs in control without any effectors was 13.77 ± 1.04 μm (n = 7). In our investigation, all calixarenes solutions contain some amount of DMSO because the first stock solutions of any calixarenes were prepared in pure DMSO. According to the results, 1.25% DMSO, which presented in 50 μM solutions of any calixarenes, didn't affect EHD of the SMCs (Fig. 8), therefore, the effects of calixarenes didn't depend on the DMSO. 100 nM solution of oxytocin served as positive control of contractile activity which decrease EHD of the uterine SMCs. Oxytocin is a hormone of a peptide nature, which is a well-known uterotonic and is widely used in obstetrics, and its action is based on the induction

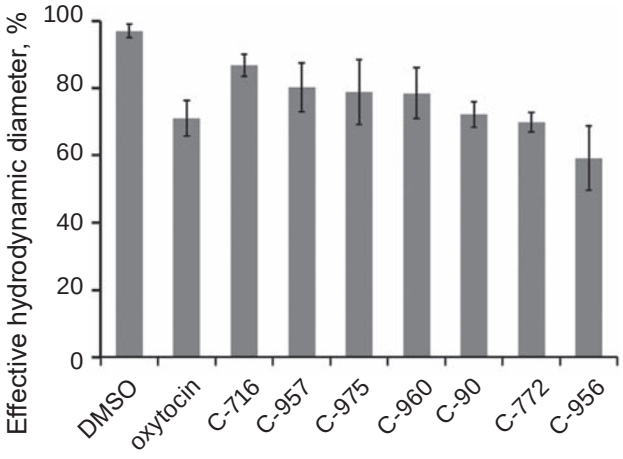


Fig. 10. Calixarenes (50 μM) similar to oxytocin (0.1 μM) decrease EHD of SMCs (n = 7). Concentration of DMSO is 1.25% (v/v). 100% is EHD without any effectors

of intracellular calcium growth through phospholipase C dependent way [30]. 100 nM oxytocin caused a decrease of EHD by 28.9 ± 5.3% compared to the control that was the evidence of SMC contraction (Fig. 10).

All calixarenes, except C-716, have a statistically significant effect on EHD of myocytes compared to control (Fig. 10). As noted above, according to enzymatic analysis, calixarene C-716 exhibits low inhibitory effect on PMCA and does not affect $[Ca^{2+}]$ of SMCs. Among the tested calixarenes, C-956 is the most effective, which at concentration of 50 μM resulted in 40.9 ± 9.5% reduction of EHD to the control (Fig. 8) that also corresponds to the results of enzymatic analysis as the most effective PMCA inhibitor (Table). It should be noted that, the decrease of EHD by the calixarene C-956 was much more significant than by calixarene C-90, which decreased SMC EHD on 27.8 ± 3.8%. In addition, the effect of calixarene C-956 was even higher than that of uterotonic oxytocin (Fig. 8). The differences in the effects of oxytocin and all calixarenesare are not statistically significant, which also indirectly indicates the ability of all tested calixarenes to reduce EHD of myocytes with efficiency depending on their structure. Calixarenes C-975, C-957 and C-960 caused almost the same decrease of EHD to 78.8 ± 9.6%, 80.2 ± 7.3 and 78.4 ± 7.6 respectively, that less than effect of calixarenes C-90 and C-956 and also correspond to efficiency of PMCA inhibition by them (Table). The only calixarene, whose effect on EHD of SMCs doesn't coincide with property to inhibit PMCA, is C-772 (Fig. 2 and 10). Moreover, this calixarene and also C-716 don't induce growth $[Ca^{2+}]$ in SMCs that can be explained by other mechanisms of influence on SMC size. Anyway, all calixarenes, which inhibit PMCA, decrease also EHD of SMCs similar to oxytocin that can be the evidence of SMC contraction effects.

Conclusions

1. PMCA inhibition efficiency depends on quantity of sulfonylamidine groups on the upper rim of calixarene macrocycle.

2. PMCA inhibition efficiency depends on spatial orientation of sulfonylamidine groups on the upper rim of calixarene in case of two sulfonylamidine-group presence.

3. *Tert*-butyl residues on the upper rim of calixarene don't effect on the efficiency of the PMCA inhibition, but prevent growth of $[Ca^{2+}]$ in SMCs.

4. Growth of hydrophobicity sulfonylamidinocalixarene due to octyl chains at the lower rim or phenyl rings connected to sulfo groups slightly increase efficiency of PMCA inhibition.

5. Almost all calixarenes, which inhibit PMCA, stimulate growth of intracellular $[Ca^{2+}]$ in SMCs.

6. All calixarenes stimulate decreasing of size of SMCs and such effect mostly correlates with their efficiency of PMCA inhibition.

All obtained results and conclusions are important for understanding and subsequent investigation of mechanisms of PMCA inhibition by calixarene C-90 or its analogs and can be foundation for creation of new more effective inhibitors of mentioned enzyme or/and uterotonics for medicine, based on the calixarene core.

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ІНГІБУВАННЯ Ca^{2+},Mg^{2+}-АТРази ПЛАЗМАТИЧНОЇ МЕМБРАНИ КАЛІКСАРЕНСУЛЬФОНІЛАМІДИНАМИ. СТРУКТУРНО-ФУНЦІОНАЛЬНІ ОСОБЛИВОСТІ

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Раніше ми показали, що тетрасульфоніламідинкаліксарен С-90 інгібує Ca^{2+},Mg^{2+}-АТРазу гладенькоміязових клітин плазматичної мембрани селективно до інших АТР-гідролаз плазматичної мембрани. Для пошуку шляхів посилення ефективності інгібування Ca^{2+},Mg^{2+}-АТРази синтезовано декілька нових каліксаренових сполук, структурно подібних до каліксарену С-90, та перевірено їх вплив на активність зазначеного ензиму, рівень цитоплазматичної концентрації Ca^{2+} та гідродинамічний діаметр ізольованих гладенькоміязових клітин. Показано, що сульфоніламідинові групи є вирішальними для інгібування Ca^{2+},Mg^{2+}-АТРази, ефективність інгібування залежить від їх кількості та просторової орієнтації на верхньому вінці каліксаренового макроциклу. Введення фенільних або трет-бутильних груп у верхній вінець і довгих алкільних ланцюгів у нижній вінець призвело до незначного підвищення ефективності інгібування. Інгібуюча дія досліджуваних каліксаренів на Ca^{2+},Mg^{2+}-АТРазу корелювала з впливом на цитозольну концентрацію Ca^{2+} та гідродинамічний діаметр гладенькоміязових клітин. Отримані результати важливі для створення більш ефективних та селективних інгібіторів Ca^{2+},Mg^{2+}-АТРази плазматичної мембрани як регуляторів скоротливої функції гладеньких м'язів.

Ключові слова: Ca^{2+},Mg^{2+}-АТРаза плазматичної мембрани, внутрішньоклітинна концентрація Ca^{2+}, гладенькі м'язи, каліксарен сульфоніламідини.

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