

Selective inhibition of smooth muscle plasma membrane transport Ca^{2+} , Mg^{2+} -ATPase by calixarene C-90 and its activation by IPT-35 compound

Iuliia I. Mazur¹, Tetyana O. Veklich¹, Oleksandr A. Shkrabak¹, Nikolai A. Mohart², Anatoly M. Demchenko², Inna V. Gerashchenko², Roman V. Rodik³, Vitaly I. Kalchenko³ and Sergiy O. Kosterin¹

¹ Palladin Institute of Biochemistry of NAS of Ukraine, Kyiv, Ukraine

² Government agency “Institute of Pharmacology and Toxicology of NAMS of Ukraine”, Kyiv, Ukraine

³ Institute of Organic Chemistry NAS of Ukraine, Kyiv, Ukraine

Abstract. We investigated the influence of calixarene C-90 and IPT-35 on plasma membrane Ca^{2+} -pumping ATPase (PMCA), intracellular calcium homeostasis and myometrium smooth muscle strain contractions. It has been shown that both effectors (100 μM) affect PMCA enzymatic activity: calixarene C-90 inhibits it by 75% and IPT-35 activates it by 40%. These compounds don't affect the Mg^{2+} -ATPase, Mg^{2+} -independent Ca^{2+} -ATPase and Na^+ , K^+ -ATPase enzymatic activities. C-90 inhibition coefficient $I_{0.5}$ magnitude was approximately 20 μM and the Hill coefficient n_H was 0.55. For IPT-35 activation, constant $A_{0.5}$ was 6.4 and n_H was 0.7. Mathematical modeling demonstrated the implication of calixarene C-90 on unexcited myocytes, which allows for a precise change in cytoplasm Ca^{2+} concentration and an influence on basal muscle tonus. By the same method, we determined that IPT-35 has a little influence on Ca^{2+} concentration in unexcited myocytes. It was also shown that calixarene C-90 *in vitro* can increase velocity of oxytocin-initiated contractions, whereas IPT-35 can suppress this aforementioned parameter. These results are promising for the design of new pharmacological compounds as better regulators of uterine contractions. Calixarene C-90 can be used in obstetric cases for the simultaneous use of oxytocin for enhancing uterine contractions, and IPT-35 for its antispasmodic effect on uterine tone.

Key words: PMCA — Smooth muscle cell — Calixarene — Cyclopenta[d]pyrimidine derivative

Introduction

Smooth muscles (SM) promote functioning of internal organs and their systems (Barany 1996; Shmidt and Tevsa 2005; Guibert et al. 2011). SM activity depends on one of the key events in cell life – changing of intracellular calcium ion concentration (Aguilar and Mitchell 2010; Iino 2010; Santo-Domingo and Demareux 2010). The uterus is the largest SM organ, as it performs special functions in gestation and labor. Uterine contractility can be changed by influence of environmental factors (sex and hypophysis hormones, mediators,

ions, mechanical stretching, and others) (Buxton 2004, 2007; Aguilar 2010). Investigation of cellular mechanisms involved in the control of myometrium contractility is important for a better understanding of obstetric pathology; for example, this would include premature and complicated labor, as well as post-labor bleeding etc, and for improving of treatment methods for these pathologies.

Spontaneous action potentials that spread on entire myometrium provoke periodical synchronous intracellular Ca^{2+} concentration elevations, which in turn control myometrium contractility and tone. Ca^{2+} concentration changes induce contraction/relaxation in response to some agents. The Mg^{2+} -ATP-dependent plasma membrane (PM) calcium pump (transporting Ca^{2+} , Mg^{2+} -ATPase or PMCA, the Ca^{2+} activation constant $K_{\text{Ca}} \approx 0.3\text{--}0.4 \mu\text{M}$) participate in intracellular Ca^{2+} concentration regulation, which influences myometrium

Correspondence to: Oleksandr Shkrabak, Palladin Institute of Biochemistry of NAS of Ukraine, Leontovycha str. 9, Kyiv 01601, Ukraine

E-mail: sashashkrabak32@gmail.com

contractions. It is important to thoroughly investigate peculiarities of PMCA activity regulation by compounds that can selectively change them. Moreover, direct changes in PMCA activity can elucidate the role of this protein in intracellular Ca^{2+} concentration regulation in normal and pathological conditions. It will likely be possible to correct myometrium activity dysfunctions by targeting changes in PMCA activity.

Nowadays there are no well-known low molecular weight effectors which selectively regulate PMCA activity. In our previous work, we found that two compounds – calixarene C-90 was the selective inhibitor of PMCA activity (Veklich et al. 2014), while another compound, IPT-35, activated the Ca^{2+} -transport activity of Ca^{2+} , Mg^{2+} -ATPase of PM. Therefore, in this work, we will continue to investigate calixarene C-90 and IPT-35 effects on SM cell PMCA and then attempt to compare them. The above compounds comprise a promising platform for the design of selective modifiers, which can be used for targeting PMCA activity regulation, and pharmacological drugs in medical practice (Consoli et al. 2006; Demchenko et al. 2009; Kononevych et al. 2011).

Material and Methods

Synthesis and structure of calixarene C-90 and IPT-35

Calixarene C-90 (5,11,17,23-tetra(trifluoro)methyl(phenylsulfonylimino)-methylamino-25,26,27,28-tetrapropoxycalix[4]arene) was synthesized and characterized by infrared spectroscopy and nuclear magnetic resonance in Phosphoranes Chemistry Department in Institute of Organic

Chemistry of NAS of Ukraine (Rodik et al. 2005). Structure of the mentioned compound is shown at Fig. 1.

Cyclopentane[d]pyrimidine derivate IPT-35 was synthesized in Department of Synthesis of Physiologically Active Compounds (in GA “Institute of Pharmacology and Toxicology of NAMS of Ukraine”). Its structure is also shown at Fig. 1 (Demchenko et al. 2009; Kononevych et al. 2011).

Physiological methods

Physiological investigations were performed in Department of Cardiovascular Agent Pharmacology (in GA “Institute of pharmacology and toxicology of NAMS of Ukraine”).

Longitudinal muscle strips were cut out from uterine horns after rat decapitation. Animals were narcotized by diethyl ether regarding to International guiding principles for biomedical research involving animals (1985 year).

Obtained strips were fixed in flow chamber on two steel hooks with load of 10 mN. Chamber volume was 0.5 ml and it was perfused by Ringer’s solution (velocity 1.7 ml per min). Temperature was constant: 37°C.

Contraction power was measured in isometric regime by capacitive strain sensor (FTK-0.1; PLC «Miosensor», Russian Federation). Contraction registration was on PC using programs DataTrax2 and LabScribe2, and analog-digital Lab-Trax-4/16 (World Precision Instruments).

Contraction records of longitudinal muscle strips from isolated rat uterine horns were performed after stabilization period – about 40 min in flow chamber. Input data were recorded for 10 min after obtaining stable phase contractions of isolated SM preparations. Then investigated compounds

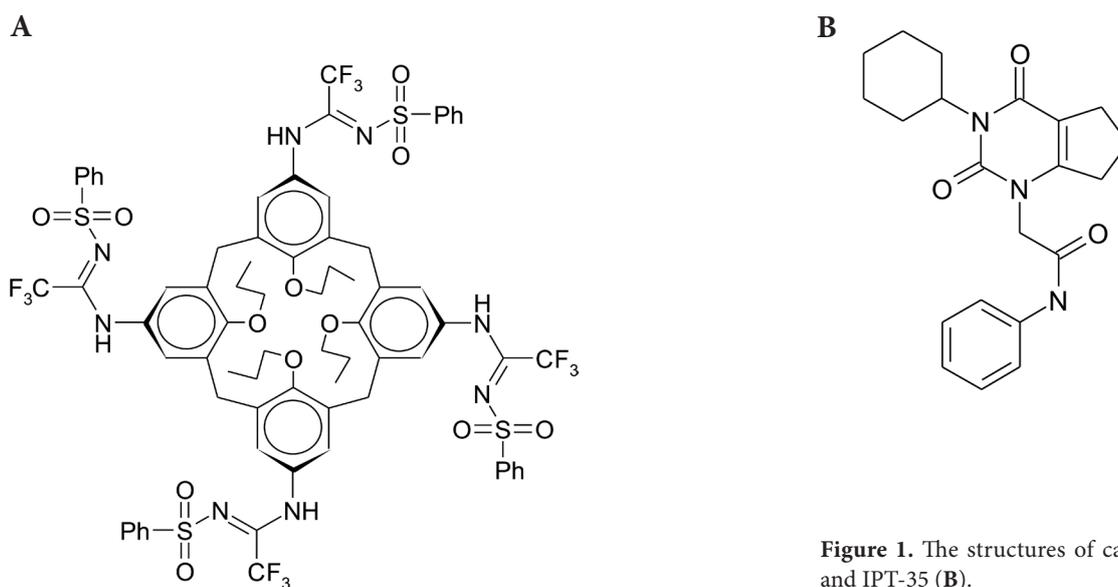


Figure 1. The structures of calixarene C-90 (A) and IPT-35 (B).

(100 μM) were added for 15 min, whereafter oxytocin was added (0.1 IU/ml).

Normalized maximal oxytocin-initiated contraction velocity was determined on contraction records by the method described in (Burdyga and Kosterin 1991). Group with intact myometrium was used as a control.

Biochemical investigations

Biochemical investigations were carried out in Department of Muscle Biochemistry in Palladin Institute of Biochemistry of NAS of Ukraine.

Preparative chemistry

Experiments were carried out in accordance with the European Guidelines and International Laws and Policies (86/609/EEC). All procedures conformed to the guidelines of the Palladin Institute of Biochemistry. Before starting the experiments, the protocols were approved by the Animal Care and Use Committee of the Palladin Institute of Biochemistry (Protocol No.1 from 21/04/2014).

Uterus smooth muscle cell PM fraction was isolated from swine myometrium as was previously described (Konratuk et al. 1986; Veklich and Kosterin 2005). Protein concentration in membrane fraction was determined by method (Bradford 1976) using reaction with Coomassie dye G250.

Enzyme investigations

General ATPase activity was determined in PM fraction of myometrium cells. Temperature was 37°C, incubation medium (volume 0.4 ml) was (in mM): 3 ATP, 3 MgCl_2 , 0.95 CaCl_2 , 25 NaCl, 125 KCl, 1 EGTA, 20 HEPES-tris-buffer (pH 7.4), 1 NaN_3 (mitochondrial ATPase inhibitor (Flynn et al. 2001)), 1 ouabain (selective inhibitor of Na^+ , K^+ -ATPase (Valente et al. 2003; Wang et al. 2004)), 0.1 μM thapsigargin (sarcoplasmic reticulum Ca^{2+} , Mg^{2+} -ATPase selective inhibitor (Flynn et al. 2001; Doan et al. 2015)), and 0.1 % digitonin (PM perforation agent (Veklich et al. 2002)). Free Ca^{2+} concentration in incubation medium in case of stable physico-chemical parameters was estimated by «MAXCHEL» program. Thus, in mentioned constant physico-chemical conditions and ion concentrations free Ca^{2+} concentration was calculated to be 1 μM .

Ca^{2+} , Mg^{2+} -ATPase activity was estimated as a difference between ATPase activity in case of Ca^{2+} presence and absence in the incubation medium.

Mg^{2+} -ATPase activity was determined in myometrium cell PM fraction in incubation medium (temperature 37°C, volume 0.4 ml) with following concentrations: 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. “Basal” Mg^{2+} -ATPase activity estimated as difference between P_i in incubation medium in presence and absence of PM fraction with correction for endogenous P_i in membrane fraction.

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

Ca^{2+} -ATPase also was revealed in uterus myocyte PM. This ATPase differs from Ca^{2+} , Mg^{2+} -ATPase because its activity can be detected in the presence of Ca^{2+} and ATP in mM concentrations and absence of Mg cations (Magoosi and Penniston 1991; Mikhailova et al. 1992). Ca^{2+} -ATPase has a low affinity for Ca^{2+} – activation constant K_{Ca} is 1 mM (Magoosi and Penniston 1991). Mg^{2+} -dependent Ca^{2+} -ATPase activity with low affinity was determined in myometrium cell PM fraction in medium (temperature was 37°C, volume 0.4 ml) with following concentration (in 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. Mentioned Ca^{2+} -ATPase activity was calculated as difference between P_i in incubation medium in presence and absence of PM fraction with correction for endogenous P_i content in membrane fraction.

In all experiments protein quantity in membrane fraction was 20–30 μg , duration of incubation was 5 min. Enzymatic reaction was initiated by addition to incubation medium PM solution aliquot (50 μl), stopped by addition of 1 ml “stop”-solution with following concentration: 5 M sodium acetate, 3.7% formaldehyde, 14% ethanol, 5% trichloroacetic acid, pH 4.3 (at 8°C). Quantity of reaction product P_i was determined by method (Rathbun and Betlach 1969).

In experiments the average magnitudes of PM Ca^{2+} , Mg^{2+} -ATPase, Na^+ , K^+ -ATPase, Mg^{2+} -ATPase, and Mg^{2+} -independent low affine Ca^{2+} -ATPase activities were: 3.4 ± 0.3 , 10.2 ± 0.7 , 18.1 ± 1.2 and 12.7 ± 2.0 $\mu\text{M P}_i/\text{mg}$ of protein *per* hour ($n = 7$), respectively.

In order to study the influence of calixarene C-90 and IPT-35 in different concentrations on ATPase activities of PM fraction we used incubation mediums described above. Solutions (20 mM) of calixarene and IPT-35 in DMSO were diluted by water to obtain required concentration of agents.

Mathematical modeling of intracellular calcium concentration in unexcited myocytes

For this purpose program (“Groovy”) was composed, which made calculations with presented precision.

Kinetic analysis

Inhibition coefficient $I_{0.5}$ and Hill coefficient n_H were calculated from kinetic curves of calixarene concentration depend-

ency influence on enzyme activity. For this purpose linearized Hill graphs were used according to equation: $\lg[(A_o - A)/A] = -n_H \lg I_{0.5} + n_H \lg [C-90]$, where A_o and A are specific enzyme activity in case of absence (“zero point”) and presence of calixarene C-90 in incubation medium in concentration $[C-90]$.

Likewise, activation coefficient $A_{0.5}$ and Hill coefficient n_H were calculated from linearized Hill graphs according to equation: $\lg[(A_{max} - A)/(A - A_0)] = n_H \lg A_{0.5} - n_H \lg [IPT-35]$, where A_o and A are specific enzyme activities in case of absence (“zero point”) and presence of IPT-35 in incubation medium in concentration $[IPT-35]$, A_{max} is maximal specific enzyme activity in case of maximal IPT-35 concentration (100 μ M).

Statistical analysis

Obtained data were statistically analyzed with Student's t-criterion. All the experiments were repeated minimum five times ($n = 5$) and the results are presented as mean \pm standard error. Kinetic and statistic calculations were made with MS Excel.

Reagents

In work the following reagent were used: ATP, HEPES, ouabain, thapsigargin, fluo-4 AM, Hoechst (“Sigma”, USA), Tris-(hydroxymethyl)aminomethane (“Reanal”, Hungary), digitonin (“Merck”, Germany), EDTA (“Fluka”, Switzerland), oxytocin (PA “Pharmstandart-Biolik”, Ukraine), heparin (PLC “Novopharm Biosintes”, Ukraine). Others were of domestic production of analytical and chemical purity.

Results

In this work, we studied the effect of calixarene C-90 and IPT-35 on ATPase activities, and free Ca^{2+} concentration changes in smooth muscle cells and myometrium contractions.

Calixarene C-90 and IPT-35 as effectors of uterine myocytes PMCA

Promising results can be observed with derivatives of phenol macrocycle – calixarene C-90. In the concentration of 100 μ M, it inhibits PMCA activity to $25.1 \pm 0.5\%$, relative to control (Fig. 2A). It should be emphasized that in this concentration, calixarene barely had an effect on ATPase activity of Mg^{2+} -ATPase, Ca^{2+} -ATPase, and Na^+, K^+ -ATPase: residual ATPase activities were $107.7 \pm 1.0\%$, $99.3 \pm 5.51\%$, and $94.2 \pm 0.6\%$, respectively (Fig. 2A) (Veklich et al. 2014).

Among derivatives of cyclopenta[d]pyrimidine and imidazo[1,2- α]azepine (such as IPT-35, IPT-176, IPT-208, and IPT-221), which engage in tocolytic activity, IPT-35 seemed to selectively activate PMCA activity (Mochort et al. 2014). This compound in the concentration of 100 μ M increased PMCA enzyme activity to $140.9 \pm 0.4\%$ (Fig. 2B). Regarding other PM ATPases, IPT-35 had the below effect on residual activities of Mg^{2+} -ATPase, Ca^{2+} -ATPase, and Na^+, K^+ -ATPase: $105.5 \pm 1.0\%$, $95.0 \pm 1.5\%$, and $96.8 \pm 0.6\%$, respectively (Fig. 2B).

Thus, two compounds efficiently and selectively influenced enzyme PMCA activity (on the PM level): in the concentration of 100 μ M calixarene, it is inhibited by 74.9%; in the same concentration, IPT-35 activates PMCA by 40%,

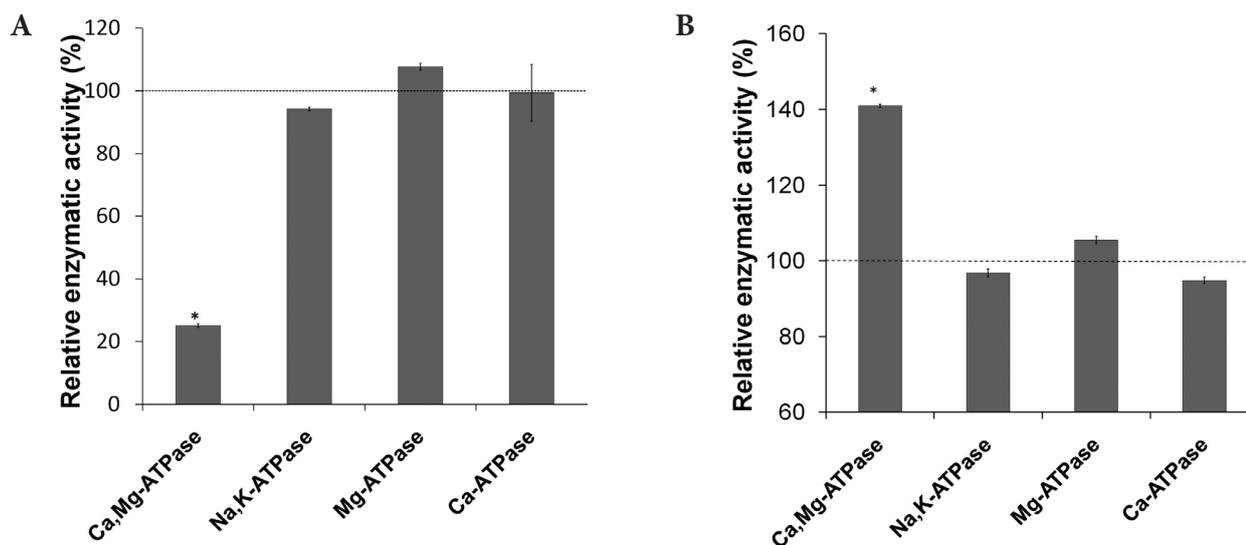


Figure 2. Calixarene C-90 (100 μ M) (A) and IPT-35 (100 μ M) (B) selectively change (inhibits and activates respectively) PMCA activity of myometrium cells. Data are presented as mean \pm standard error ($n = 5$). 100 % is an enzyme activity without of calixarene C-90 or IPT-35 in incubation medium (control). * $p < 0.05$ compared to Na^+, K^+ -ATPase, Mg^{2+} -ATPase and Ca^{2+} -ATPase.

relative to control. Both of these compounds do not influence other PM ATPase activities.

In other experiments, we analyzed PMCA activity inhibition dependence on the concentration of calixarene C-90 (10^{-8} – 10^{-4} M) (Fig. 3A). In this concentration range, calixarene C-90 efficiently (in a dose-dependent manner) suppressed ATPase activity. The calculated inhibition coefficient $I_{0.5}$ was 20.2 ± 0.5 μ M that is the evidence of sufficient affinity of calixarene C-90 to the enzyme. The Hill coefficient was 0.55 ± 0.02 (Table 1) (Veklich et al. 2014).

The same analysis was made for IPT-35. Results revealed that IPT-35, in a dose-dependent manner activated activity of PMCA (Fig. 3B). Its activation constant $A_{0.5}$ was 6.4 ± 0.45 μ M, with the Hill coefficient $n_H = 0.7 \pm 0.04$ (Table 1).

Calixarene C-90, compared to IPT-35, is a more efficient effector of PMCA enzyme activity when both compounds are examined in the same maximal concentration. Change of ATPase activity by C-90 action was 5-fold (inhibition), while for IPT-35, it was only 1.4 (activation) (Fig. 3).

Kinetic model of calixarene C-90 and IPT-35 influence on intracellular calcium homeostasis in unexcited uterine myocytes

Simulation of calixarene C-90 (selective PMCA inhibitor) affected equilibrium intracellular Ca^{2+} concentration in unexcited myocytes

We have previously shown that calixarene C-90 causes a decrease in enzyme turnover, or the apparent maximal velocity $\langle V_{max} \rangle$ of PMCA reaction in control (without calixarene in incubation medium), which was 6.7 μ mol

Table 1. Kinetic characteristics of calixarene C-90 and IPT-35 actions on PMCA activity of myometrium cells

Parameters	C-90	IPT-35
$I_{0.5}$ (μ M)	20.2 ± 0.5	—
$A_{0.5}$ (μ M)	—	6.40 ± 0.45
n_H	0.55 ± 0.02	0.70 ± 0.04
Inhibition (%)	74.9 ± 0.5	—
Activation (%)	—	40.9 ± 0.4

Data are presented as mean \pm standard error ($n = 5$). $I_{0.5}$, inhibition coefficient; $A_{0.5}$, activation coefficient; n_H , Hill coefficient.

P_i /hour per mg of protein, then dropping off in the case of calixarene action. The PMCA apparent activation constant $\langle K_{Ca} \rangle$ without calixarene in incubation medium was 190 ± 1 nM, with the Hill coefficient $n_H = 2.1$. Application of calixarene C-90 in a concentration of 0.1–10 μ M had no influence on PMCA's apparent activation constant by Ca^{2+} $\langle K_{Ca} \rangle$ and the Hill coefficient n_H for Ca^{2+} . Further increase of C-90 concentration (> 50 μ M) caused significant changes in kinetic parameters: $\langle K_{Ca} \rangle$ increased to 312 nM, and the Hill coefficient n_{Ca} decreased to 1.5. Thus, only a high calixarene concentration (50–100 μ M) could induce reduction in PMCA affinity for Ca^{2+} and decrease the positive cooperative effect of PMCA activation by Ca ions (Veklich et al. 2013). Considering all determined kinetic parameters of calixarene C-90 inhibitory action on PMCA enzyme activity, we have elaborated a quantitative permanent model of intracellular calcium concentration which changes in uterine myocytes in the case of effector application.

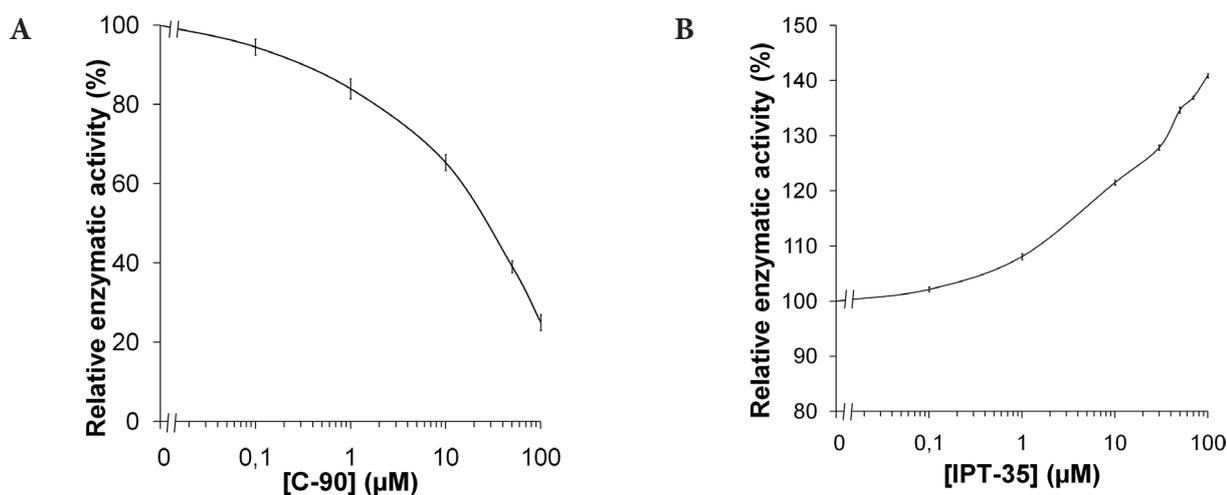


Figure 3. Dependence of PMCA activity on calixarene C-90 (A) and IPT-35 (B) concentrations. Data are presented as mean \pm standard error ($n = 5$). 100 % is an enzyme activity without calixarene C-90 or IPT-35 in incubation medium (control).

It was shown (Veklich et al. 2013) that calixarene C-90's influence on PMCA velocity can be described by the Hill equation:

$$V = \frac{\langle V_{max} \rangle [Ca^{2+}]_i^n}{\langle K_{Ca} \rangle^n + [Ca^{2+}]_i^n} \quad (1)$$

where $\langle V_{max} \rangle$ is apparent maximum velocity of Ca^{2+} extrusion from the cell across the membrane, $[Ca^{2+}]_i$ is concentration of intracellular Ca^{2+} , $\langle K_{Ca} \rangle$ is apparent activation constant for Ca^{2+} , n is Hill coefficient for Ca^{2+} .

According to recent data, PMCA is responsible for maintaining Ca^{2+} homeostasis and preventing its increase. It counteracts input of Ca^{2+} flow, which is caused by the Ca^{2+} transmembrane gradient between cytoplasm and an outside medium (Strehler et al. 2007; Oloizia and Paul 2008). Taking into account that the Hill coefficient n_{Ca} of PMCA is approximately equal to 2 (Veklich et al. 2013), changing the cytoplasm Ca^{2+} concentration in unexcited uterine myocytes can be described by the following differential equation:

$$\frac{d[Ca^{2+}]_i}{dt} = \gamma([Ca^{2+}]_e - [Ca^{2+}]_i) - \frac{\langle V_{max} \rangle \times [Ca^{2+}]_i^2}{\langle K_{Ca} \rangle^2 + [Ca^{2+}]_i^2} \quad (2)$$

Here, the first part of the right-hand side describes input flow of Ca^{2+} in the cell across PM, caused by the gradient ($[Ca^{2+}]_e - [Ca^{2+}]_i$) and depending on the membrane's permeability constant (particularly for Ca^{2+}) γ . The second part involves the velocity of PMCA Ca^{2+} -transport activity. In the case of a permanent condition, in which Ca^{2+} concentration

in the cell is constant, this means that $(\frac{d[Ca^{2+}]_i}{dt}) = 0$. As such, Eq. 2 can be rewritten as:

$$\gamma[Ca^{2+}]_i^3 + (\langle V_{max} \rangle - \gamma[Ca^{2+}]_e) \times [Ca^{2+}]_i^2 + \gamma \langle K_{Ca} \rangle^2 [Ca^{2+}]_i - \gamma \langle K_{Ca} \rangle^2 [Ca^{2+}]_e = 0. \quad (3)$$

Solving Eq. 3 for $[Ca^{2+}]_i$ we obtain dependence of basal $[Ca^{2+}]_i$ in unexcited myocytes on constant of PM permeability γ , Ca^{2+} concentration in extracellular medium $[Ca^{2+}]_e$, the apparent activation constant for Ca^{2+} $\langle K_{Ca} \rangle$ and apparent pump velocity $\langle V_{max} \rangle$. This equation has three roots: one has a negative value, while the others are coupled, with the value of positive one corresponding to the expected intracellular Ca^{2+} concentration (≈ 100 nM).

Given that C-90 caused the decrease of PMCA V_{max} , it was assumed that in concentrations of 0.1–50 μ M, calixarene acted as a full non-competitive inhibitor of PMCA. Using principles of enzyme kinetics, the following equation is obtained:

$$\langle V_{max} \rangle = \frac{I_{0.5}}{I_{0.5} + [C-90]} V_{max} \quad (4)$$

The fixed values of $\gamma = 10^{-3} \text{ sm}^{-1}$ (Oloizia and Paul 2008), $[Ca^{2+}]_i = 10^{-3} \text{ M}$ (Kosterin 1990), $K_{Ca} = 1.8 \times 10^{-7} \text{ M}$ and $I_{0.5} = 2 \times 10^{-5} \text{ M}$ were used, while V_{max} was simulated by a model, with an initial baseline of $4.6 \times 10^{-6} \text{ M/min}$ (Kosterin 1990). As a result, according to Eqs. 3 and 4, dependence of basal equilibrium Ca^{2+} concentration in unexcited uterus cells on calixarene C-90 (selective PMCA inhibitor) was obtained (Fig. 4A).

From this result, we may assume that the action of calixarene C-90 on $[Ca^{2+}]_i$ has two phases: C-90 concentration 1–25 μ M provokes the slow increase of intracellular Ca^{2+} concentration. Application of this compound in a specific concentration allows us to change precisely the Ca^{2+} concen-

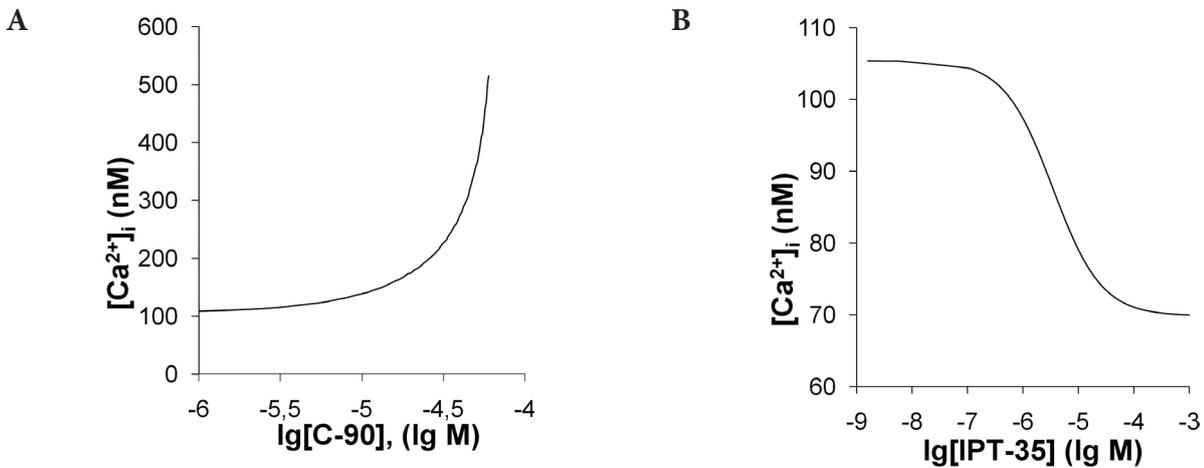


Figure 4. Simulated dependence of balanced Ca^{2+} concentration in unexcited myometrium cell on calixarene C-90 (A) and IPT-35 (B) concentrations. According to Eq. 3, estimated basal intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ is 105 nM.

tration and gently influence its basal muscle tonus. Application of calixarene C-90 in concentrations higher than 25 μM causes an exponential increase of basal Ca^{2+} concentration, which can yield SM contracture.

Simulation of IPT-35 (selective PMCA activator) effect on equilibrium intracellular Ca^{2+} concentration in unexcited myocytes

As seen in our results, IPT-35 had no influence on the Ca^{2+} activation constant K_{Ca} – while it increased enzyme maximum hydrolysis velocity (data not shown); therefore, according to principles of enzyme kinetics, IPT-35 was accepted as a non-competitive PMCA activator. This indicates that Ca^{2+} and IPT-35 interacts with PMCA independently, creating a ternary complex and increasing the velocity of ATP hydrolysis. Maximal apparent PMCA velocity $\langle V_{\max} \rangle$ change was described by the following expression:

$$\langle V_{\max} \rangle = \frac{K_A + \beta[IPT - 35]}{K_A + [IPT - 35]} V_{\max} \quad (5)$$

where K_A is the activation constant ($A_{0.5} = 6.4 \mu\text{M}$, Table 1), and β ($\beta = 1.31$) is the ratio of k_2/k_2' in the Botts and Morales mechanism.

Likewise, using Eq. 3 in the case of the calixarene C-90 model and Eq. 5, we obtained the dependence of Ca^{2+} concentration in unexcited myometrium cells on IPT-35 concentration (Fig. 4B). According to this model, IPT-35 causes the decrease of Ca^{2+} concentration by 35%, relative to its initial level, as it is the plateau level which is insensitive to the additional effector concentration increase.

Compared to calixarene C-90, an activator of PMCA – IPT-35 is a mild effector, and its application does not cause significant change of Ca^{2+} concentration in myocytes (Fig. 4B). The action of IPT-35 on $[\text{Ca}^{2+}]_i$ can be described as a sigmoid curve, with the most effective concentration being 0.3–3 μM .

Calixarene C-90 and IPT-35 influence on myometrium contraction kinetics

In the study on isolated rat longitudinal muscle strips, it was calculated that normalized oxytocin-induced (0.1 IU/ml) contraction velocity V_{nc} (control) was $0.0205 \pm 0.0024 \text{ s}^{-1}$ ($n = 10$). For calixarene C-90 preincubation (100 μM during 15 min), the normalized oxytocin-induced contraction velocity V_{nc} increased two-fold up to $0.0438 \pm 0.0014 \text{ s}^{-1}$ ($n = 18$) (Fig. 5). At the same time, preincubation with IPT-35 in these conditions (100 μM during 15 min) caused a statistically significant decrease of oxytocin-induced contraction velocity V_{nc} to $0.0135 \pm 0.0005 \text{ s}^{-1}$ ($n = 10$) (Fig. 5). These results confirm the antispasmodic effect

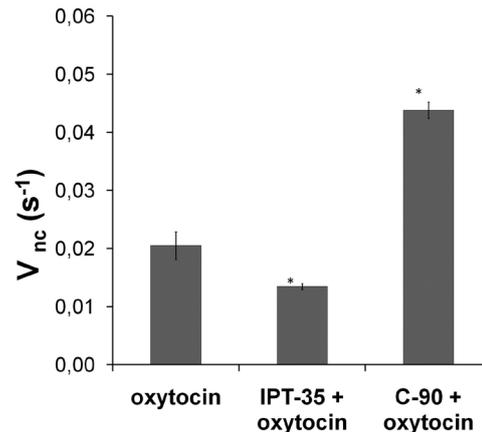


Figure 5. Application of calixarene C-90 (100 μM) and IPT-35 (100 μM) changes normalized velocity contractions V_{nc} of rat myometrium *in vitro* initiated by oxytocin (0.1 IU/ml). Data are presented as mean \pm standard error ($n = 7$). Mechanokinetic parameters were calculated by method (Burdyga and Kosterin 1991). * $p < 0.05$ compared to oxytocin.

of IPT-35 in smooth muscles *in vitro* and *in vivo* (Pupysheva 2013).

Discussion

PMCA is one of the high affinity Ca^{2+} -extrusion systems of cells (Kosterin 1990). This enzyme is part of the systems of calcium homeostasis, signal transduction, relaxation/contraction and their regulation the case of smooth muscle cells. It is important to study the properties of PMCA to understand the specific physiological value of this enzyme, its regulation and interaction with other proteins. Today the most widely-used methods are genetic manipulations with the expression of PMCA. However, as PMCA content in PM is low, there is a methodological problem in obtaining high levels of its expression and the “correct” (physiologic) integration in PM (Adamo et al. 1992; Brini and Carafoli 2009). Due to a tight functional connection between different Ca^{2+} -regulation systems, Ca^{2+} -transporters, and Ca^{2+} -sensors, they can also change their expression or activity in the case of additional PMCA transcript appearance (Liu et al. 1996). Thus, it is important to use alternative methods of PMCA investigation with agents that can selectively change their activity. It may also help avoid the compensatory effect connected with genetic manipulations.

SM cells should be regarded as a complex receptor tenselectrochemical system, which is characterized with nonlinear, cooperative, non-additional, and synergistic properties of Ca^{2+} homeostasis. Interpretation of the obtained experimental data on a mechanical level is too

difficult in this context. However, some observations can be made.

It was established that two compounds with low molecular weight – calixarene C-90 and IPT-35 may selectively (on the PM level) modify PMCA enzyme activity – to inhibit and to activate, respectively (Fig. 2). Investigated compounds are opposite in their effect on PMCA activity, so we also expect that results of their actions on smooth muscle Ca^{2+} concentration and contraction pattern can be inverse. Our obtained data confirm our assumption about the action of specific PMCA inhibitors and activators on SM cells.

Calixarene C-90 is a sufficient PMCA inhibitor ($I_{0.5} = 20.2 \pm 0.5 \mu\text{M}$, Fig. 3A) which must suppress Mg^{2+} and ATP-dependent Ca^{2+} transport across myometrium PM. Our results confirm this assumption according to the mathematical model (Fig. 4A) of how calixarene C-90 evokes an increase of Ca^{2+} concentration in unexcited myocytes, and increased oxytocin-induced myometrium contractions *in vitro* (Fig. 5). Calixarene C-90 is assumed to be a prospective agent for application simultaneously with oxytocin to increase uterine contractions.

IPT-35 is a PMCA activator ($A_{0.5} = 6.40 \pm 0.45 \mu\text{M}$, Fig. 3B), which according to the mathematical model weakly influences Ca^{2+} concentration in unexcited myocytes (Fig. 4B). Nevertheless, it suppresses oxytocin-induced myometrium contraction velocity *in vitro* (Fig. 5) indicating that IPT-35 may cause an antispasmodic effect on uterine contractions with the simultaneous use with oxytocin. Thus, IPT-35 is also perspective for a pharmacological application, because pathological conditions connected with improper uterine SM activity often require muscle relaxant application, and mild PMCA activation after influence of low effector concentration can curing muscle tone. The role of PMCA in smooth muscles is not limited to the control of muscle basal tonus by maintaining stable Ca^{2+} concentration. PMCA participates in the regulation of SM contraction parameters of oxytocin response that are indirectly confirmed by our results (Fig. 5). This may be controlled by changing local calcium signaling waves or the pattern of calcium oscillation. Nevertheless, these assumptions must be confirmed by additional experimental data.

There are no selective inhibitors or activators of PMCA besides the so-called caloxins (Pande et al. 2011) that complicate the investigation of physiological functions of this enzyme. We can conclude that obtained results may be useful for further design of new selective effectors of PMCA – on the basis of calixarene C-90 and IPT-35 for investigation of PMCA's physiological functions and pharmacological regulation of uterine contractility in pathological conditions.

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