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Nitric oxide induced polarization of myometrium cells plasmalemma revealed by application of fluorescent dye 3,3′-dihexyloxacarbocyanine

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The fluorescence probe 3,3'-dihexyloxacarbocyanine was employed to estimate NO-induced changes of myometrium cells plasma membrane and mitochondria membrane potential by the methods of flow cytometry and spectrofluorimetry. The donors of NO, sodium nitroprusside and sodium nitrite were shown to increase the plasma membrane potential. The polarization of the plasma membrane exhibited by applied nitro-compounds was appreciable at physiologically significant concentrations of extracellular Ca^{2+}. The rise of membrane potential has not been achieved as dihydropyridine-sensitive Ca^{2+}-channels were blocked by nitrendipine or nonselective inhibitors of K^+ channels, 4-aminopyridine, and tetraethylammonium. Nitro compounds-induced inhibition of high-potassium membrane depolarization was time-dependent. In conclusion, NO caused the increase in membrane potential of myometrium cells at the state of functional rest due to the stimulation of Ca^{2+}-dependent K^+-permeability of the plasma membrane and counteracted membrane depolarization thus, suggesting the increased susceptibility of myocytes to myoconstriction factors.

Keywords: Nitric oxide, Potential susceptible carbocyanine probes, Smooth muscle, Transmembrane potential, Uterus

Separate researches are conducted for studying nitric oxide (NO) role in the regulation of the contractile activity of smooth muscle uterus (myometrium) along with the final determination of its essential value in the mechanisms of relaxation of the smooth muscles of the vessels, the gastrointestinal, respiratory and urogenital tract[1]. The obtained experimental results allow us to make assumptions about the value of nitric oxide in processes that prevent a contractile response for stretching the walls of the uterus in the process of embryo growth and reduce the sensitivity of myometrium to myoconstrictor agents, which is particularly important in the normal course of pregnancy[2,3]. Either the NO product or the sensitivity to it decreases at the end of pregnancy and precede the beginning of labor activity[2,4]. NO sources can serve as an endometrial tissue and vascular endothelium in the uterus. Nerve terminals, which contain neuronal NO-synthase and provide nitrergic innervation of the uterus, are also identified[5,6].

Nitric oxide donors cause relaxation of myometrium non-pregnant women as well as those with different periods of pregnancy[7]. The corresponding decrease in contractile capacity of the uterine smooth muscle cells is also shown in the case of certain species of animals, including rats and primates, at different periods of the functional activity of the uterus[8,9]. At the same time, the physicochemical mechanisms by which nitric oxide controls the contractile function of myometrium are still not fully understood today.

The relaxing NO effect is associated with an increase in the content of cyclic guanosine-3′,5′-monophosphate (cGMP) in the myoplasm in various types of smooth muscle cells[1]. However, the value of cGMP in the relaxation of mechanical tension has been questioned in studies carried out on the myometrium of non-pregnant and pregnant women, primates, rats, and guinea pig[7,10]. An alternative direction of action of NO in the smooth muscles, including in myometrium, may have a direct effect on cation-transport systems of plasmalemma, which is carried out by nitrosylated derivatives of nitric oxide[11-13].

It is assumed that an important biochemical mechanism by which NO can cause relaxation of the uterus smooth muscle is cGMP-dependent or direct activation of the plasma membrane K^+-channels[14,15]. Several subtypes of K^+-channels have been identified in the myometrium of human and animals, particularly high and low conductivity Ca^{2+}-dependent, ATP-dependent, potential-dependent (calcium-sensitive), *etc*.[14,16-18]. Substances that

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contribute to the opening of the K^+- channels reduce the excitability of cells, causing the displacement of the membrane potential from its threshold values necessary for the activation of ion-transport systems. There is a shift of electric potential in the plasmalemma of the myometrium cells towards to more negative values during the pregnancy of a human and rats, which may be due to the strengthening of K^+-channel currents[14,16]. The probable consequence of the various subtypes K^+-channels activation by nitric oxide is plasma mem brane hyperpolarization and a decrease in the myoplasmic Ca^{2+}-concentration.

It is important to maintain intact of the subcellular membrane structures during the study of their electrical potential, which requires work on unharmed cells. This allows us to maintain the native morphology of plasmalemma and intracellular compartments, and also largely maintains functional relationships between membrane structures[19-22]. Subcellular membranes under normal conditions have significant electrical potential as a result of the work of ion transporters and electronic transport chains. The internal mitochondrial membrane, plasma membrane, first of all in the case of excitatory cells, as well as the membranes of the endoplasmic reticulum due to the activity of the microsomal cytochrome system[19-24], are significant in most animal objects. A proper study of changes in electrical potential in the case of intact cells requires consideration of the partial contribution of these membrane components, as well as the use of compounds that would be able to influence their polarization selectively.

Potential-sensitive fluorescent probes such as derivatives of rhodamine, oxonol or dyes of the cyanine series[19,21,22,25] have been used for the purpose of analyzing the changes in the polarization of subcellular structures for a long time. Cyanine dyes are widely improved into experimental practice for the study of transmembrane potentials in both plasma and mitochondrial membranes[20-24], while information on their use in smooth muscle cells is quite limited.

We have shown that in myocytes of the uterus nitric oxide increases the permeability of the plasma membrane to Ca^{2+} and stimulates its dihydropyridine-sensitive passive transport into cells in a series of the previous studies[26]. These membrane and ionic effects can cause the short-term growth of calcium concentration in the sarcolemma region, which will activate the Ca^{2+}-dependent subtypes of K^+-channels, followed by hyperpolarization of the membrane and decrease the level of myocyte excitability. The possibility of using 3′3′-dihexyloxacarbocyanine dye was used to record changes in the electrical potential on a plasma membrane in the presented paper in order to confirm this hypothesis. The effect of nitro compounds on the polarization of plasmalemma by the functional rest of myocytes and the development of artificial depolarization have been studied too.

Materials and Methods

Chemicals

In the work the following reagents were used: Hepes, glucose, saccharose, bovine serum albumin, poly-L-lysine, collagenase type IA, EGTA, $CaCl_2$, valinomycin, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) sodium azide (NaN_3), ouabain, sodium nitroprusside (SNP), sodium nitrite (SN), 4-aminopyridine (4-AP), tetraethylammonium (TEA), nitrendipine (Sigma, USA); 3,3′-dihexyloxacarbocyanine ($DiOC_6(3)$), Hoechst 33342, soybean trypsin inhibitor (Fluka, Switzerland); MitoTracker Orange CM-H2TMRos (Invitrogen, USA). Any other reagents produce in Ukraine.

Isolation of myocytes from myometrium of rats

Suspension of smooth muscle cells of the uterus of non-pregnant rats, estrogenated 16 h before tissue intake, was obtained using collagenase and soybean trypsin inhibitor by the Mollard method[27] in our modification. The narcosis of animals was caused by inhalation of chloroform after which they were subjected to decapitation. We have complied with all requirements for laboratory animals.

The tissue was cut out into small pieces (average size - 2 × 2 mm) after removing the uterus, purifying it from fat and connective tissue, and washed off from blood and high concentrations of Ca ions (3 times for 5 min) in Hanks B - solution (a solution “B”) of the following composition, (mM): NaCl - 136.9; KCl - 5.36; KH_2PO_4 0.44; $NaHCO_3$ - 0.26; Na_2HPO_4 - 0.26; $CaCl_2$ - 0.03; Hepes - 10 (pH, 7.4, 37°C); glucose - 0.1%. Pieces of tissue were incubated for 20 min (37°C, continuous mixing) in 2 mL tissue dissociation media, which was a solution of collagenase (0.1%), bovine serum albumin (0.1%) and soybean trypsin inhibitor (0.01%), prepared on solution “B”. The tissue dissociation medium was taken and the tissue was transferred to a free solution from the enzyme preparation “B” after 20 min. The tissue preparation was gently piped 15-20 times with a glass pipette for

1-2 min to accelerate the dissociation of the cells. Solution "B", containing dissociated cells, was selected and the tissue preparation was again transferred to a new portion of the cell dissociation medium, containing collagenase and a soybean trypsin inhibitor. This procedure was repeated 5-6 times. The first two portions of the solution "B" were used for pipetting and containing dissociated cells were discarded due to the fact that they contained tissue fragments and damaged cells. The last 3-4 portions were collected, combined and centrifuged for 10 min at 80 g. The resulting cell suspension was washed with solution "B" and again centrifuged in the above mode. Cells were resuspended with a solution "B".

1 mL of the received cell suspension contained an average of 6.6 million myocytes; the number of viable cells - 90-95% of the total number of cells (this characteristic has been determined with trypan blue).

Study of plasma and internal mitochondrial membranes of smooth muscle cells polarization using potential-sensitive probe $DiOC_6(3)$ by flow cytometry and spectrofluorimetry methods

Changes in the transmembrane potential of plasma and mitochondria of intact myocytes have been recorded according to the fluorescence intensity of the potential-sensitive probe of the carboxycyanine series, 3,3′-dihexyloxacarbocyanine ($DiOC_6(3)$)[28-30]. Changes in the fluorescence response of $DiOC_6(3)$ in uterine myocytes have been investigated using a flow cytometry method on a Coulter Epics XL^{TM} (Beckman Coulter, USA) with an argon laser, λ_{ex} = 488 nM, λ_{fl} = 510 nM (channel Fl1), which allows record the fluorescence signal of individual myocytes. The result of the studies is an obtaining a "number of events-intensity of fluorescence" curve, the peak position of which corresponds to the maximum of the fluorescence response of most cells in their general population. Parallel studies have been performed using the spectrofluorimetry method on the Quanta Master 40 PTI (Canada) spectrometry, λ_{ex} = 485 nM, λ_{fl} = 505 nM, which analyzes the fluorescence signal from the cell suspension. Measurements have been carried out in the physiological Hanks medium (solution "B"). Amount of cells in the sample was 0.3-0.5 million.

Visualization of membrane electrical potential of myometrium cells using laser confocal microscopy

The mutual distribution of the potential-sensitive probe $DiOC_6(3)$ and the specific for mitochondria MitoTracker Orange CM-H_2TMRos in myocytes has been observed at a dye concentration of 200 nM. The spatial localization of the dyes has been analyzed on a laser scanning confocal microscope LSM 510 META ("Carl Zeiss", Germany) using immobilized myocytes on poly-L-lysine[31]. Investigations on a confocal microscope have been conducted in Multi-Track mode. The fluorescence of Hoechst 33342 has been excited using a 405 nM wavelength laser, and the BP 420-480 filter has been used to signal registration. The excitation of the $DiOC_6(3)$ fluorescence has been carried out at a wavelength of 488 nM, and its registration has been in the range of 505-530 nM (BP filter – 505-530). Laser with a wavelength of 543 nM has been used for the MitoTracker Orange CM-H_2TMRos fluorescence excitation and its fluorescence has been recorded in the range of 560-615 nM (BP 560-615 filter).

Data are expressed as means ± SE of the numbers of determinations. Differences between paired sets of fluorimetric experiments were analysed using paired Student's *t*-tests in Microsoft Excel.

Results

Probe $DiOC_6(3)$ is a lipophilic cation which potential-dependent accumulates within a limited membrane space[19,21,25,28-30,32]. The existence of negative potential on biomembranes leads to an increase in its accumulation. In this case, first of all, the probe is redistributed between the cytosol and the mitochondrial matrix, depending on the concentration, the ratio of the probe/cell, as well as the functional state of the cells themselves. Reducing the fluorescence intensity of the probe in myocytes may indicate a decrease in the membrane polarization while its growth is due to an increase in the magnitude (modulo) of the transmembrane potential of the plasma and/or internal mitochondrial membranes or other compartments[28,29,32].

We have chosen the conditions for the registration of the fluorescent response of $DiOC_6(3)$ in the uterine myocytes using the method of flow cytometry[19,20,25,28-30]. The main advantage of this method is the ability to register the fluorescence signal of individual myocytes. Parallel studies have been conducted using spectrofluorimetry which contributes to their verification. The fluorescence signal of the suspension of myocytes in the working environment, rather than individual cells, is analyzed in the latter variant. We have applied commonly used substances depolarizing mitochondria (5 mM sodium azide, an electron transport chain inhibitor, 10 μM protonophore CCCP)[33] and plasma membrane (1 mM ouabain - a known blocker of the corresponding Na^+,

K^+-ATPase)[34]. Control experiments have shown that the autofluorescence of the cell suspension does not change during the course of the measurement, and no changes in the intensity of autofluorescence have been detected under the influence of the compounds that have been used.

We have investigated a wide range of $DiOC_6(3)$ concentrations - from 10 nM to 5 μM. The kinetics of the fluorescence signal at the interaction of the probe (10 - 500 nM) with the cells is characterized by saturation, which is achieved for about 3 min of incubation (Fig. 1A shows a typical result for the concentrations of the 100 nM probe). Its intensity is almost unchanged, for at least 10 min. It has been shown that for 10 nM $DiOC_6(3)$ the pre-incubation of myocytes (5 min) with agents that depolarize the plasma membrane (1 mM ouabain or 120 mM KCl) does not lead to changes in the fluorescence intensity. Therefore, there is no response of the probe to the electrical potential of plasmalemma (Fig. 1B). At the same time, the use of 1-5 μM $DiOC_6(3)$ results in quenching of the fluorescence of the probe (Fig. 1C, the results for the concentration of the probe 1 and 5 μM are shown). It is known that the dimerization of cyanine dyes to increase of concentration results in a fluorescence drop[21]. The optimal conditions for recording a fluorescence response in the case of uterine myocytes are $DiOC_6(3)$ concentrations in the range of 50-100 nM.

Reducing the fluorescence of myocytes in the previous incubation with ouabain, azide and the CCCP (Figs. 1A & 2A) suggests the possibility for

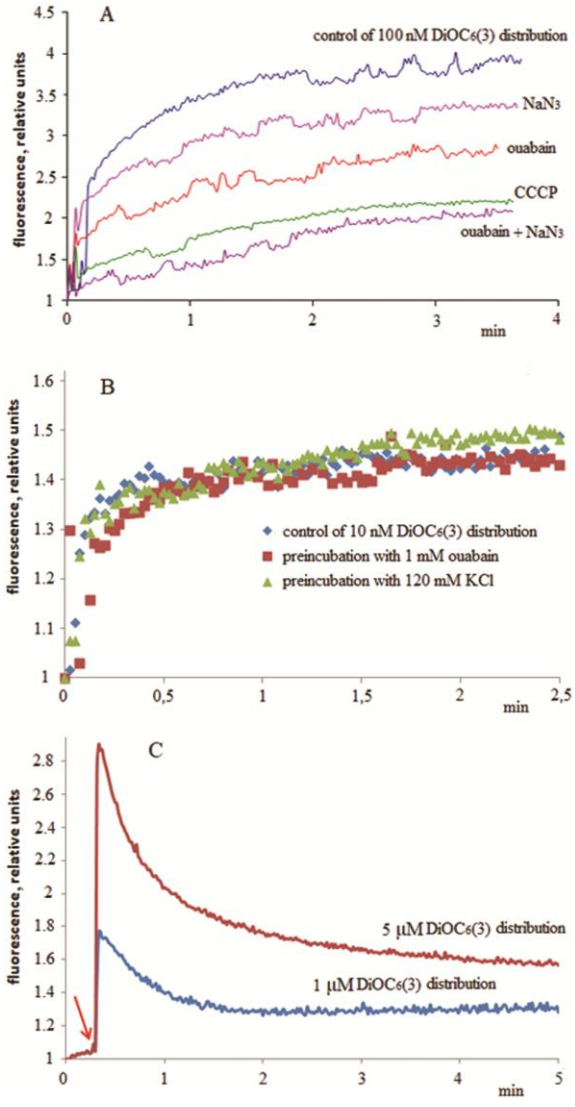

Fig. 1A — Fluorescent response of 100 nM, B - 10 nM, 1 and C - 5 μM, $DiOC_6(3)$ in uterine myocytes. The results were obtained using a spectrofluorimeter. Concentrations of substances: 1 mM ouabain, 10 μM CCCP, 5 mM NaN_3. Preincubation of cells with substances was 5 min (A & B). The result of a typical experiment. At least 5 independent experiments were conducted

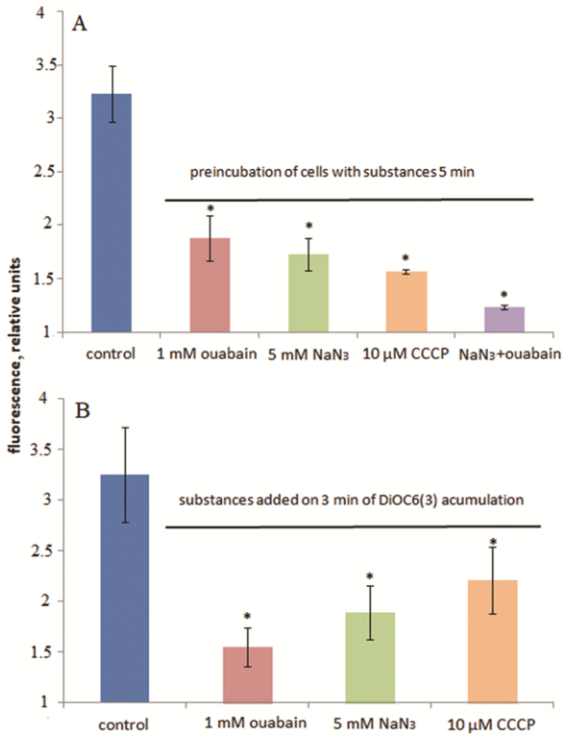

Fig. 2 — Fluorescent response of 100 nM $DiOC_6(3)$ in uterine myocytes in the presence of depolarizing agents of plasmalemma and mitochondria: A - Preincubation of cells with substances was 5 min (fluorescence relative units are given 5 min after $DiOC_6(3)$ distribution); B - The transit addition of agents depolarizing the membrane of mitochondria and plasmalemma (fluorescence relative units are given 5 min after the added of substances). **P* <0.05, Student's *t*-test as compared to the control. Data are shown as mean of 5 independent measurements±SD. The results were obtained using a flow cytometer. For 1 taken cells' autofluorescence

testing potentials both in the plasma membrane and in the mitochondria of the cells. The total effect of quenching the fluorescence signal in the presence of sodium azide and ouabain in the applied concentrations in some cases is 90% (Fig. 2A). Protonophore CCCP and NaN_3 suppress the fluorescence response of cells also with the addition on the 3 min accumulation of $DiOC_6(3)$, *i.e.* at the plateau level (Fig. 2B). The fluorescence response is reduced by almost 2-fold after 5 min of their action. A similar effect in the direction is observed with the addition of ouabain. The intensity of fluorescence decreases more than 2 times after 5 min (Fig. 2B). Data, obtained both with the use of spectrofluorimetry and flow cytometry, has had a qualitatively similar character (Figs. 1 & 2).

It has been shown, using laser confocal microscopy, that $DiOC_6(3)$ is accumulated in the cell volume possibly bind to mitochondria and, interacting with a charged plasma membrane, outlines the cell contours (Fig. 3A). The comparative distribution of the potential-sensitive $DiOC_6(3)$ and specific for mitochondria MitoTracker Orange CM-H_2TMRos in the uterine myocytes indicates their colocalization (Fig. 3B). The latter dye accumulates in energized organelles, which have a significant negative potential on the inner membrane, and covalently binds to intra-mitochondrial proteins[35]. Hence, $DiOC_6(3)$ interacts with the plasma membrane and is also accumulated in the mitochondria of the uterine myocytes.

K^+-ionophore valinomycin leads to an increase in the $DiOC_6(3)$ fluorescence of myocytes (Fig. 4A). Valinomycin has enhanced the fluorescence response of the probe in the case of the internal membrane of mitochondria has been depolarized, which has been provided by the pre-incubation of cells for 5 min with 1 mM sodium azide. It's a consequence of an increase in the permeability of the plasma membrane to the

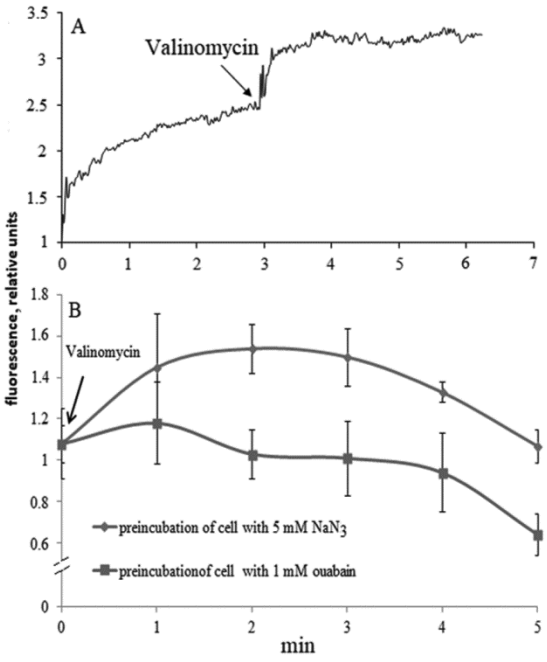

Fig. 4 — The action of K^+-ionophore valinomycin on the fluorescence of $DiOC_6(3)$ in uterine myocytes. A - Effect of 0.5 μM valinomycin on the total fluorescence of $DiOC_6(3)$ (spectrofluorometry method). For 1 taken cells' autofluorescence. Data of a typical experiment. At least 5 independent experiments were conducted. B - Effect of 0.5 μM valinomycin on fluorescence $DiOC_6(3)$ under preincubation of cells with 5 mM NaN_3 or 1 mM ouabain (flow cytometry method). The arrow indicates the moment of adding an aliquot of the solution of the valinomycin. Data are shown as mean of 5 independent measurements±SD

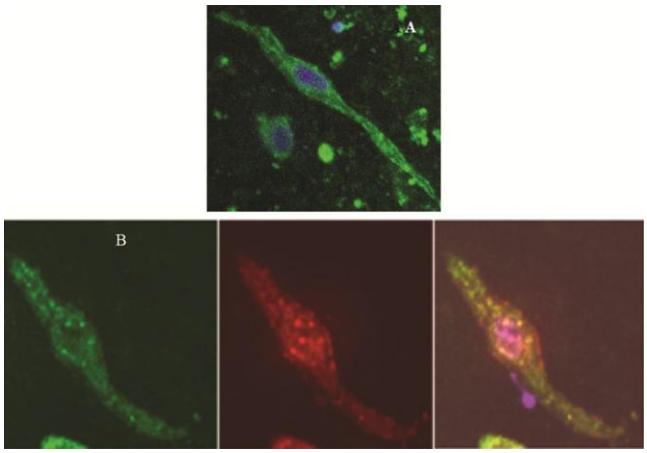

Fig. 3 — Distribution of fluorescent probes in uterine myocytes. A - distribution of fluorescent dyes $DiOC_6(3)$ (green), a lens with 63× magnification; B - green - $DiOC_6(3)$, red - MitoTracker Orange CM-H_2TMRos (mitochondria specific), overlay of both images; purple - Hoechst 33342 (nucleus specific). The result of a typical experiment. At least 5 independent experiments were conducted

K ions and corresponding increase (in modulus) of the transmembrane potential (Fig. 4B). At the same time, plasma membrane depolarization lead to the pre-incubation of myocytes with 1 mM ouabain makes it not sensitive to K^+-ionophore. The decrease of the $DiOC_6(3)$ fluorescence may indicate the depolarization of the internal mitochondrial membrane due to an increase in its K^+ permeability under these conditions (Fig. 4B).

Thus, studies have shown that $DiOC_6(3)$ fluorescence probe and flow cytometry or spectrofluorimetry methods may be used to analyze changes in the electrical potential on a plasma membrane of myocytes under conditions of depolarization of the internal mitochondrial membrane (pre-incubation of cells with NaN_3 or CCCP). These results allow us to study the effect of nitric oxide on the polarization of the plasma membrane of myometrium cells provided that 1 mM sodium azide is present in the incubation medium. It is precisely these conditions that we have used in the experiments.

The effect of nitro-compounds namely sodium nitroprusside (SNP) and sodium nitrite (SN) on rat uterine myocytes at rest demonstrates the growth of plasma membrane polarization. This effect is significantly dependent on the Ca ions presence in the medium incubation of cells (Fig. 5). The polarizing effect of nitro compounds is almost not observed for low (non-physiological) concentrations of Ca ions or the presence of Ca^{2+}-chelator EGTA. The SNP and SN effects depend on their concentration and are not seem in the submicromolar range (Fig. 6).

Non-selective K^+-channels inhibitors tetraethylammonium (TEA) and 4-aminopyridine (4-AP) lead to a steady decrease in the polarization of the plasma membrane (Fig. 7A & B), while the inhibitor of dihydropyridine-sensitive Ca^{2+}- channels nitrendipine have not significantly affected the plasma membranes in our variant of the experiment (Fig. 7C). Adding to myocytes of the abovementioned compounds action 0.1 mM SNP and SN for 7 min have not led to an increase in the electrical potential in the plasmalemma. Further reduction of the membrane potential has been observed in the case of depolarization of the plasma membrane by tetraethylammonium (Fig. 7D), as well as by the action of 4-aminopyridine in the case of SN (Fig. 7E). Pre-treatment of myocytes with nitrendipine almost completely has inhibited hyperpolarization of plasmalemma by the action of nitro compounds (Fig. 7F), although in the case of SN there has been a slight tendency to increase the fluorescence of $DiOC_6(3)$.

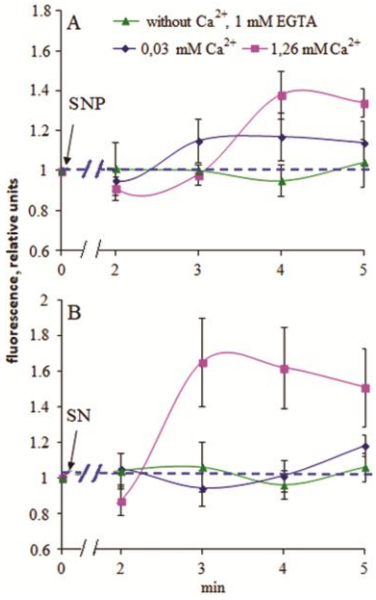

Fig. 5 — Polarization of myometrium cells plasmalemma was caused by 0.1 mM SNP (A) and SN (B) in presence 1 mM EGTA, 0.03 mM Ca^{2+} and 1.26 mM Ca^{2+}. To eliminate the effect of mitochondria, the cells were preincubated for 5 min with 5 mM sodium azide. For 1 taken cells' fluorescence on 3 min of accumulation $DiOC_6(3)$. Data are shown as mean of 6 independent measurements±SD

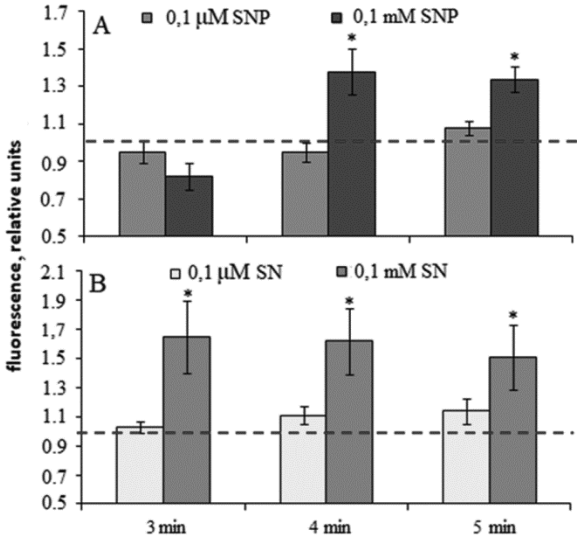

Fig. 6 — Polarization of myometrium cells plasmalemma was caused by 0.1 μM or 0.1 mM SNP (A) and SN (B) in presence 1.26 mM Ca^{2+}. To eliminate the effect of mitochondria, the cells were preincubated for 5 min with 5 mM sodium azide. **P* <0.05, Student's *t*-test as compared to the control of the absence of nitro compounds. Data are shown as mean of 6 independent measurements±SD. For 1 taken cells' fluorescence on 3 min of accumulation $DiOC_6(3)$

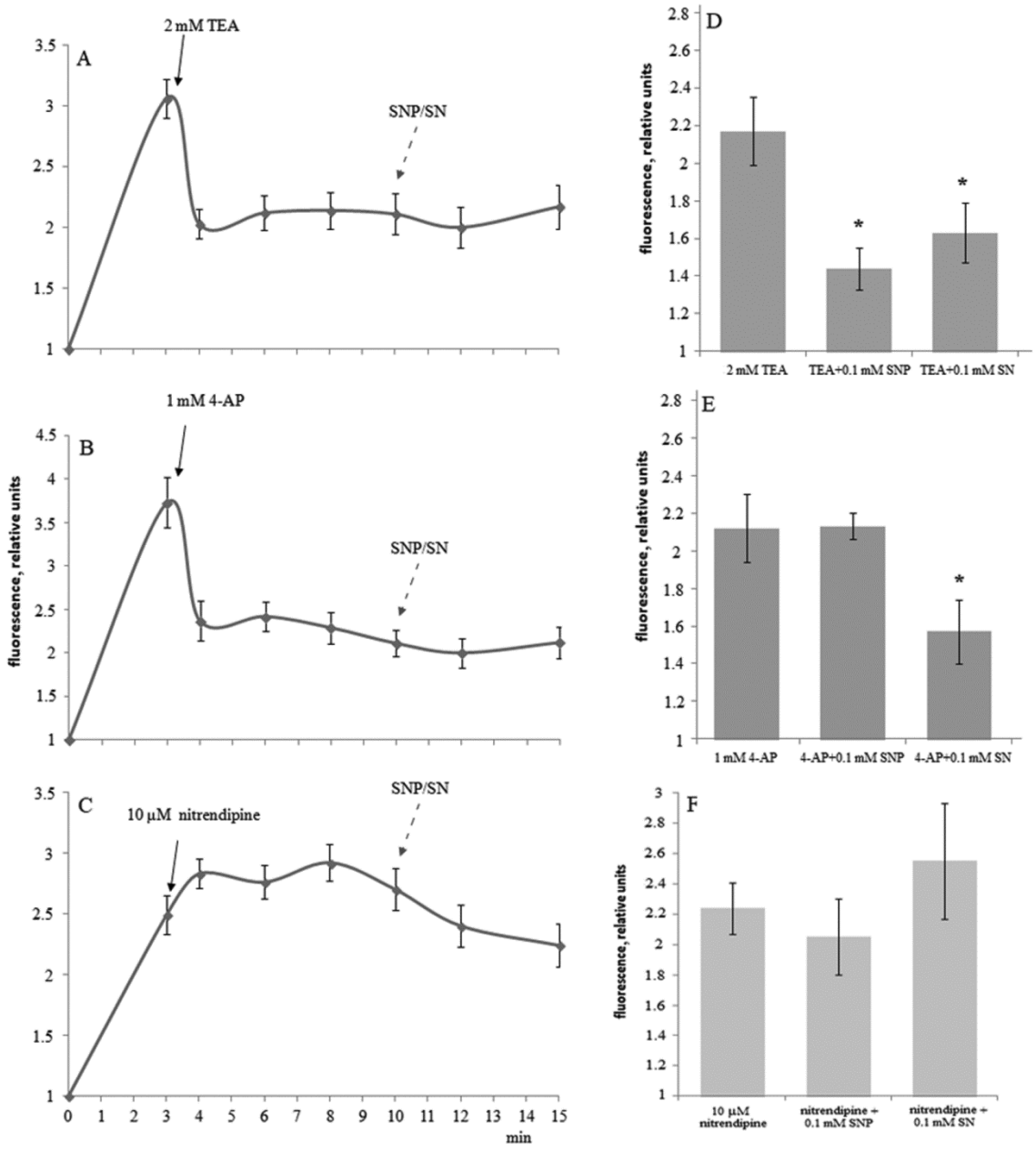

Fig. 7 — Changes in the polarization of uterine myocytes plasmalemma by the action of K^+-conduction blockers TEA (A) and 4-AP, (B) 0.1 mM SNP and SN (D, E), and also in the presence of a specific dihydropyridine Ca^{2+}-channel inhibitor nitrendipine (C & F). SNP and SN transit added on 7 min of inhibitor action. A - C -control changes in polarization; D - F - fluorescence relative units are given 15 min (after 5 min the introduction of substances). The black arrow indicates the moment of adding TEA, 4-AP, nitrendipine at control conditions; the red dotted arrow indicates the moment of adding SNP or SN at experimental conditions. **P* <0.05, Student's *t*-test as compared to the control of the absence of nitro compounds. Data are shown as mean of 5 independent measurements±SD. To eliminate the effects of mitochondria, the cells were preincubated for 5 min with 5 mM sodium azide. For 1 taken cells' autofluorescence

The effects of nitric oxide on the development of uterine myocytes depolarization over time have been also studied. High-potassium depolarization has been used as a model when 100 mM NaCl has been isotonically replaced by 100 mM KCl in a standard physiological medium. It has been established that myocyte pre-incubation for 15 min with 0.1 mM SNP or SN does not affect the initial depolarizing effect of isotonic replacement of NaCl on KCl, but the development of high-potassium depolarization in time is effectively suppressed by the investigated compounds (Fig. 8).

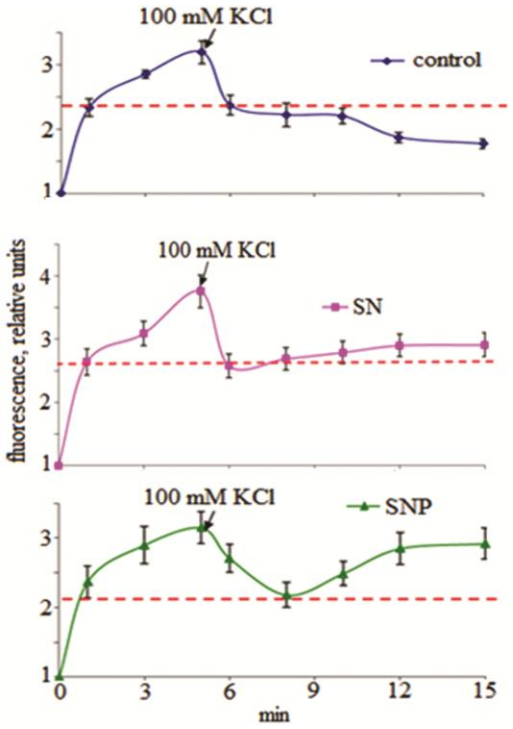

Fig. 8 — Influence of 0.1 mM SNP and SN on the development of high-potassium depolarization in the time (preincubation of uterine myocytes for 15 min with nitro compounds). To eliminate the effects of mitochondria, the cells were preincubated for 5 min with 5 mM sodium azide. Data are shown as mean of 5 independent measurements±SD. For 1 taken cells' autofluorescence

Discussion

It has been proved that one of the potential-sensitive probes of the carbocyanine series $DiOC_6(3)$ can be used to evaluate the changes in plasma membrane polarization under artificial conditions destruction of the potential on the internal mitochondrial membrane in the case of working on fresh intact myocytes of the rat uterus during work with the use of spectrofluorimetry, flow cytometry and laser confocal microscopy, as well as known modifiers of the electrical potential of subcellular membrane structures. The optimal conditions for the practical use of this probe on the investigated object have been selected. This approach has been used to study the effect of nitric oxide on the electrical potential of the plasma membrane of non-excited uterine myocytes and in the case of their artificially depolarized. It has been shown that nitro compounds, commonly used donor (SNP) and precursor NO (SN), cause hyperpolarization of plasmalemma, which can be based on Ca^{2+}-dependent activation of K^+-channels, and counteract the development of high-potassium depolarization over time. These results have broadend our understanding of the physicochemical mechanisms that underlie the smooth muscle relaxation by the action of nitric oxide and, in particular, the increase of the threshold of excitability of the uterine myocytes under the conditions of increasing levels of nitric oxide in the myometrium.

A comparative study by spectrofluorimetry and flow cytometry methods of changing in the polarization of the plasma and internal mitochondrial membranes of freshly isolated uterine smooth muscle cells

The smooth muscle cells are relatively small in size, making it difficult to apply micromanipulation techniques to test the electric potential of the plasmalemma. In addition, the use of microelectrodes damages cell membranes. Therefore, the development of appropriate techniques using fluorescent probes is ongoing. They allow the research on intact cells to maintain the integrity of membrane compartments[21,22]. Potential-sensitive fluorescent dyes of the cyanine series are widely used for the study of transmembrane electrical potentials on both the plasma and the internal mitochondrial membranes number of cellular objects in particularly[19,20,23,25,30,32]. At the same time, there is very limited information about their use in the case of smooth muscle cells, especially uterine myocytes.

The transport of H^+ from the matrix occurs in the intermembrane space of the mitochondria, which determines the creation and maintenance of the protons electrochemical gradient on their inner membrane, which electrical component can reach the magnitudes −160 - −180 mV as a result of the respiratory substrates oxidation and the functional activity of the I, III and IV electron transport chain complexes[33]. At the same time, the electrical potential (resting potential) of the plasma membrane due to K^+-diffusion in myometrium cells is about −35 mV in non-pregnant animals[36]. The key role in providing the resting potential belongs to the Ca^{2+}-dependent K^+-channels[14,17,18]. It has been important to demonstrate with the use of $DiOC_6(3)$ that plasmalemma and mitochondria of freshly-isolated myometrium cells in our experimental conditions have a time-stable electrical potential. It has been turned out that both relatively low (10 nM) and high (1, 5 μM) concentrations of the probe are ineffective when working with myocytes. At optimal concentrations of $DiOC_6(3)$, which has been showed 50-100 nM, within 3 min of accumulation and

distribution of the probe in the cells an equilibrium state is achieved (Fig. 1), and the fluorescent response of the dye does not change with time.

The accumulation of lipophilic cations from the environment into the cytoplasm depends on the electrical potential of the internal membrane of mitochondria in intact cells, which determines the distribution of the probe between the cytosol and the mitochondrial matrix, and the magnitude of the potential of the plasma membrane, which, in turn, causes its distribution between the cytosol and the extracellular medium[19,20,23,25,30,32]. Consequently, both an electric charge of plasma membrane and the mitochondria determine the fluorescence value at a given concentration of the probe. We can study the contribution of both potentials (both plasmalemma and mitochondria) by creating the optimal probe/cell ratio in the experiment[19], and to avoid quenching the fluorescence of the probe associated with its aggregation. It has been used $DiOC_6(3)$ concentrations from 10 to 500 nM[20,23,25,30] to test the electrical potential on plasmalemma and/or mitochondria on different cell objects. The concentration 500 nM (0.1 μg/mL) of the probe has been used to estimate the potential changes precisely in mitochondria, while at its high concentrations (5 - 50 μM) the organelles swell, lose electrical charge and normal morphology. Using $DiOC_6(3)$ as a potential indicator on mitochondria, it should be taken into account that probe can act as an inhibitor of NADH dehydrogenase, like other lipophilic carbocyanines, *i.e.* its effect is rotenone-like[37]. Sometimes $DiOC_6(3)$ is used to study the electrical potential of endoplasmic reticulum membranes, but at much higher concentrations of 5-50 μM (1-10 μg/mL)[24]. There is evidence that cyanine dyes can depolarize the plasma membrane[21]. Regarding smooth muscle cells, there no relevant information in literature available for us.

The conducted studies have established that the fluorescence response of $DiOC_6(3)$ in the uterine myocytes includes the partial components associated with both the electrical potential of plasmalemma and mitochondria. The fluorescence signal is particularly sensitive to the depolarization of the plasma membrane, provided that the ouabain is treated, as well as to the depolarization of the internal mitochondrial membrane by pre-incubation of cells with sodium azide or protonophore CCCP. In addition, there is an additivity in the response of $DiOC_6(3)$ to pre-incubation of myocytes with azide and ouabain, according to data confirmed by spectrofluorimetry and flow cytometry (Figs. 1A & 2A). It's characteristically, that the fluorescence of a potential-sensitive dye is largely determined by the electrical potential of plasmalemma and mitochondria, which indicates an insignificant contribution of the electron transport chain of the sarcoplasmaic reticulum. It should also be noted that the response of $DiOC_6(3)$ to the investigational chemical agents is rather rapid (Fig. 2B), which simplifies the procedure for investigating the polarization of the plasma membrane (in the presence of sodium azide or the CCCP) and mitochondria (in the presence of ouabain) in intact myocytes. The obligatory condition for correct testing of the plasma potential in our further studies has been the pre-incubation of myocytes with sodium azide, which makes impossible contribute of the mitochondria electrical potential to the fluorescence response of $DiOC_6(3)$.

Confocal microscopy data (Fig. 3) confirms the fact that $DiOC_6(3)$ interacts with the plasma membrane and mitochondria. Although the probe interacts intensively with mitochondria due to the larger area of the internal mitochondrial membrane and significantly higher negative charge, as the colocalization of $DiOC_6(3)$ and MitoTracker Orange CM-H_2TMRos has evidenced, our results for the existent ouabain-sensitive component of the $DiOC_6(3)$ fluorescence signal and clear delineation cell contours with this dye indicate the possibility of recording changes in the electrical potential on the plasma membrane of myocytes. This conclusion is confirmed independently in experiments with valinomycin (Fig. 4). The treatment of myocytes with potassium ionophore valinomycin, which results in increased plasmalemma permeability to K^+ and hyperpolarization, leads to an increase in the fluorescence of $DiOC_6(3)$.

Thus, the fluorescence potential-sensitive probe $DIOC_6(3)$ and the method of flow cytometry can be used to evaluate the polarization of both the plasma membrane and mitochondria in intact uterine myocytes. Both membrane compartments have an electrical potential that persists for a certain time, according to our experimental conditions, which may indicate the native nature of freshly isolated myocytes. It is appropriate to use a more reliable and unambiguous method of flow cytometry in order to analyze its changes, the data of which are confirmed by spectrofluorimetry. However, the results of the

latter approach may be influenced by the physical and chemical processes that occur in the suspension of myocytes.

Taking into account the fundamental importance of changes in the electrical potential of the plasma membrane in the processes of regulating the transport of Ca^{2+}, the contractile activity and the appearance of myometrium non-excitability in the case of high content of nitrogen oxide in this tissue, we have focused our attention on the study of the plasma membrane polarization level under the action of nitro compounds using $DiOC_6(3)$ and flow cytometry.

The effect of nitro compounds on the polarization of the myometrium cellular plasma membrane and its modulation with Ca^{2+} and K^+ channel blockers

As nitro compounds, which are SNP and SN, used donor and precursor NO, widely applied in biological researches. SNP forms nitrosonium cation in solutions, which exerts the properties of a powerful nitrosylated agent. SN produces nitric oxide predominantly in an acidic medium but is capable of forming NO and its redox derivatives also in the presence of heme groups and iron-sulfur complexes that rich cellular structures, especially mitochondria[11-13,38]. In addition, nitrite anions can have their own biochemical activity.

Analyzing the results presented in (Figs. 5 & 6), it is necessary to draw attention to several facts. The increase of electric potential of the plasma membrane in the action of nitro compounds does not take place or is not significant in the presence of a non-physiologically low concentration of extracellular Ca^{2+} or in its nominal absence in the medium with a chelator. Hence, the effect of polarization of plasmalemma has a pronounced Ca^{2+}-dependent character. We have demonstrated in previous studies, that nitro-compounds increase the passive permeability of the plasma membrane to Ca ions and inhibite the activity of transporting Ca^{2+}, Mg^{2+} -ATPase (PMCA) in the myometrium[26] by the cGMP-independent manner. Both effects are capable to increase of subsarcolemma Ca^{2+} concentration and activate of Ca^{2+}-dependent processes here.

Passive transport of Ca^{2+} in the plasma membrane of the uterine myocytes is largely due to the functioning of potential-operated L-type Ca^{2+}-channels, which are dihydropyridine sensitive[39]. The dependence of the polarizing effect of nitro compounds (Fig. 7F) on the presence of nitrendipine indicates the possible involvement of these structures in the increase of the permeability of the membrane to the Ca ions. The activity of PMCA in the smooth muscle is associated with caveolae. One of the splice variants of the neuronal isoform NO synthase (nNOSμ) is colocalized with this transport enzyme. The decrease of the latter activity is accompanied by an increase in biosynthesis NO and *vice versa*[40]. It can be assumed that the Ca^{2+}-dependent increase in the synthesis of nitric oxide in the subsarcolemma region will lead to a change in the functioning of cation transport systems located here, and in particular, the corresponding subtypes of K^+-channels. The latter will lead to an increase in the electrical potential of the plasma membrane. The inhibition of K^+-penetration of plasmalemma by non-specific K^+-channel inhibitors has resulted in its pronounced depolarization in our studies (Fig. 7A & 7B), in the presence of which the hyperpolarizing effect of nitro compounds has not been manifested (Fig. 7D & 7E).

Consequently, the polarizing effect of nitric oxide is due to the activation of the K^+-penetration of plasmalemma and depends on Ca ions, suggesting that it is associated with the K^+_{Ca} channels. The rise of the plasma membrane K^+-permeability is considered an important factor in maintaining the relative non-excitability of myometrium during pregnancy in the case of rest condition[14].

Depolarization of the plasma membrane of myometrium cells in the presence of nitro compounds

The possible *in vivo* value of nitric oxide as an agent inhibiting the processes of excitation of the uterus smooth muscle is demonstrated by experiments on the effects of SNP and SN on the development of high-potassium depolarization of the plasma membrane (Fig. 8). The addition of high K^+ concentration to cells - isotonic replacement of Na^+ with K^+ in the incubation medium has resulted in a dramatic decrease in the fluorescence of $DiOC_6(3)$ in these studies, which lasted for at least 10 min in the experiment. These data indicate a depolarizing effect of high concentration of K^+ on myocytes. Their preliminary pretreatment with nitro compounds does not affect the initial development of depolarization, but the further falling of the electric potential on plasmalemma undergoes a significant inhibition. The polarization of the membrane for 15 min, according to the changes in the fluorescence $DiOC_6(3)$, becomes close to the control values (up to the time of high-potassium depolarization) in the case of SNP (Fig. 8). These effects can be explained by cGMP-dependent stimulation of Na^+, K^+-ATPase,

which we have demonstrated earlier in myometrial sarcolemma[26], with subsequent activation of the K^+-permeability of the plasma membrane by nitrogen oxide.

Conclusion

Hence, we have experimentally proved the possibility of employing the flow cytometry method to study the effect of physiologically active compounds on the transmembrane potential of both mitochondria and the plasma membrane in isolated cells of the smooth muscle of the uterus using a potential-sensitive $DiOC_6(3)$ probe. The electrical potential of subcellular structures has been quite stable in time. The results obtained using this methodological approach show that nitrogen oxide causes Ca^{2+}-dependent polarization of myometrium cells plasmalemma, which is associated with the activation of the K^+-permeability of the membrane, as well as the ability to counteract depolarizing action. These membrane and ionic effects can cause relative myocyte non-excitability under the condition of increased concentration of nitric oxide in the uterus tissues and provide resistance to myoconstrictor agents.

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