The Relation of Different-Scale Membrane Processes Under Nitric Oxide Influence

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Abstract. Rhythmic activity, synaptic transmission and propagation of excitation depend on the processes that occur at different cellular levels. Modulation of neuron activity results from the changes of plasma membrane and organelles properties. We aimed to study the relationship between changes of the ion channels activity, membrane microviscosity and amount of bound Ca^{2+} under the influence of nitric oxide (NO). We also investigated the effect of NO on the refractive index of neurons. We have shown that NO activates voltage-dependent $\text{K}(\text{K}_V)$ -channels and leads to the decrease in the amount of membrane-bound and stored Ca^{2+} . NO causes a decrease in the microviscosity of the membrane of cytosomes and altered refractive index of neurones. The latter can result from the modification of plasma membrane physico-chemical properties and structural changes in the cytoplasm (vesicles movement, reorganisation of cytoskeleton, etc.). It is suggested that long-term modifications of the physico-chemical properties of membranes and reorganisation in the cytoplasm affect neuron activity and signal transmission.

Key words: neuron, nitric oxide, membrane processes, redistribution of calcium

1. Introduction

The function of the nervous system is based on cell-to-cell communications and processes that occur in the neuronal network. All these phenomena depend on the properties of individual neurons. Modulation of neuron activity is based on its electrophysiological properties and cascades of cytoplasmic reactions that are regulated by secondary messengers. So, a neuron's response to stimuli is formed by interrelated reactions and processes distributed over long time scales and in space. Under certain conditions even genes participate in memory formation and education [1–3]. It is evident that plasma membrane processes influence the cytoplasmic reactions and *vice versa* [4–6]. In many cases it is impossible to pick out a primary process that triggers all others, but we can study connections between various spatial and different time-scale processes. In this paper we report our data on the relation of processes that occur in the plasma membrane and organelles' membranes under the influence of nitric oxide.

Figure 1. The scheme of nitric oxide influence on nerve cells. Nitric oxide (NO) affects activity of persistent sodium channels and NMDA receptors and activates soluble guanylate-cyclase (s-GC). NO inhibits production of ATP, decreases activity of Ca^{2+} -ATPase of endoplasmic reticulum (ER) and causes Ca^{2+} exit from ER and mitochondria.

Nitric oxide (II), or NO, is one of the neurotransmitters and intracellular messengers in the nervous system. Depending on the system region, NO increases or decreases the level of neuron activity, influences the synaptic transmission and propagation of excitation [7–12]. NO affects neuron function on different levels (Figure 1). In the plasma membrane NO activates persistent sodium channels and inhibits NMDA-receptors [13–16]. Inside the neuron, NO activates soluble guanylate-cyclase (s-GC) that produces a cascade of reactions with a c-GMP messenger. c-GMP activates protein kinases G and A, which modulate activity of many cytoplasmic enzymes and membrane proteins [17, 18]. In addition, NO can inhibit production of ATP by mitochondria through the reaction with Fe-containing enzymes of the respiratory chain [19, 20]. Nitric oxide also evokes redistribution of free cytoplasmic Ca^{2+} due to the decrease in the activity of Ca^{2+} -ATPases of endoplasmic reticulum (ER), depletion of ER Ca^{2+} -stores and the release of Ca^{2+} from mitochondria [21–23].

A question of great interest is the effect of NO on various types of ion channels, particularly K^{+} -channels, that determine many neuron properties. Besides, there is a lack of information on how NO influences membrane physico-chemical properties, which affect the activity of receptors, channels and ATPases.

In the present work we aim to study the influence of nitric oxide on the activity of voltage-dependent K^{+} (K_V)-channels, the amount of the membrane- and protein-bound Ca^{2+} and membrane microviscosity. We also study the relation of NO-induced processes that take place in the plasma membrane, intracellular membranes and in the cell as a whole.

2. Materials and Methods

2.1. NEURAL PREPARATION

The preparations were ganglia and isolated neurons of the pond snail *Lymnaea stagnalis*. We used motoneurons from the buccal and pedal ganglia of the pond snail except B2, B7 neurons, which form NO-ergic synapses [24]. In the cells we used, NO is not a neurotransmitter, but serves as intra- and extracellular messenger and regulator. Isolated ganglia were put in the Petri dishes with standard physiological solutions for snails (in mM: 50 NaCl, 1.5 KCl, 4 CaCl₂, 1 MgCl₂, 11 HEPES, pH 7.5). Neurons were isolated by removing the ganglion capsule with slight pressure of a thin needle on the ganglion.

For patch-clamp experiments ganglia were put into the 3.5 mg/ml solution of pronase E for 20–30 min at 37 °C. After that neurons were placed into the physiological solution for 2 h [25, 26]. Microelectrodes were filled by 10 mM KCl solution with pH 7.5.

For Raman spectroscopy we used peripharyngeal ganglia complex without buccal ganglia that contain B2, B7 neurons with NO-ergic synapse.

All experiments were performed at the room temperature. As a NO-donor we used sodium nitroprusside (SNP) (Sigma) which generates two forms of nitric oxide: NO and NO⁺. The ultimate concentrations of SNP in the experiments were 0.1–1 mM. At least 5 repeats were made for all experiments.

2.2. METHODS

Ion channel activity was recorded using the patch-clamp technique in inside-out configuration. Snail motoneurons, after incubation in the pronase E and physiological solutions were put into the experimental chamber. The physiological solution in the chamber was replaced by 100 mM KCl solution (pH 7.5) after the formation of GΩ contact between microelectrode and neuron. The membrane patch was formed by the withdrawal of the microelectrode from the cell. The recording of channel activity was performed only for voltage-dependent K⁺-channels. We tested channels for voltage-dependence, recorded channels activity and then changed 100 mM KCl solution for 100 mM KCl solution with 0.1 mM SNP. SNP solution was replaced by 100 mM KCl solution in 20 min. Ion channel activity was recorded with a patch-clamp amplifier (Institute for Biological Instrumentation, Pushino, Russia).

The amount of the bound Ca²⁺ was estimated by fluorescent microscopy with a fluorescent dye chlortetracycline (CTC) (Sigma) [27–30]. Motoneurons were incubated in the 10^{−4} M solution of CTC for 20 to 60 min. Under such conditions CTC penetrates into the plasma membrane or intracellular membranes and internal hydrophobic Ca²⁺ stores, respectively. Thus, a separate study of the amount of Ca²⁺ bound on the outer and inner membranes and Ca²⁺ stores is possible by using CTC. After the incubation in the CTC solution, neurons were transferred to the Sigard-laid Petri dishes with physiological solution. The CTC fluorescence

Figure 2. Raman spectrum of carotenoids in neurons of the pond snail *L. stagnalis*. The band with maximum position at 1526 cm^{-1} corresponds to the vibrations of C=C bonds, the band with maximum position at 1160 cm^{-1} to the vibrations of C–C bonds and the band with maximum position at 1008 cm^{-1} to the vibrations of C–CH₃ bond of –CH₃ group.

was excited with light at a wavelength of 400 nm and recorded with a Lyumam I–3 luminescence microscope, FMEL-1A photometric attachment and interference filters with a 520 nm wavelength of maximal light transmission. The SNP solution was added after the stabilisation of CTC fluorescence. The ultimate concentration of SNP was 1 mM.

Microviscosity of intracellular membranes was estimated by resonance Raman spectroscopy (RRS). Neurons of many invertebrates including snails have specific organelles called cytosomes (or carotenoidosomes), whose membrane contains molecules of C40-carotenoid [31]. Figure 2 represents Raman spectrum of carotenoids in the neurons of the pond snail.

Carotenoids are natural probes for membrane microviscosity [32, 33]. Changes of structuring of the membrane phospholipids alter the degree of conjugacy along π -electron chain of carotenoid, which is reflected in the contribution of the double C=C bond peak to the whole carotenoid spectrum. Thus, the order of fatty acid tails in the carotenoids' surroundings can be estimated by the intensity ratio of double C=C bond peak (maximum position at 1526 cm^{-1}) to single C–C bond peak (maximum position at 1160 cm^{-1}) (I_{1526}/I_{1160}) [32, 34]. It was shown that changes of the membrane viscosity obtained by electron paramagnetic resonance (EPR) spectroscopy correlate with changes of ratio I_{1526}/I_{1160} [32, 35]. Besides, membrane carotenoids are sensitive to the amount of the membrane-bound Ca^{2+} and *trans*-membrane potential [34, 36, 37].

Isolated ganglia were put into the capillary with physiological solution. After that, a laser beam of 473 nm wavelength was focused on the ganglia and registrations of the control spectra and spectra after SNP addition were performed. The ultimate concentration of SNP in the capillary was 0.5 mM. For all experiments the respective control spectra and spectra after SNP addition were averaged. The power and intensity of the laser radiation were 20 mW and $1\text{--}2\text{ kG/m}^2$, respectively. Scattered radiation was collected on the aperture of the monochromator DFS-24.

Figure 3. The principle of interference microscopy. Light beam from the laser source (L) is divided on the splitter (S). One of the beams (reference beam) reflects from the reference mirror (M2) and goes to the detector (D). The other beam transmits through an object, reflects from the mirror M1, once more time transmits through the object, goes to the detector, and interferes with the reference beam.

The monochromator had the following parameters: spectral region 400–850 nm, relative slit 1:5.3, dispersion 0.45 nm/mm, half band of apparatus function 1 cm^{-1} at 550 nm, scan rate 0.018 nm/s, resolution 4 cm^{-1} . Raman spectra were recorded on the FEY-79 photomultiplier operating in the photon counting mode. Spectra accumulation was performed using multichannel analyser NTA 1024.

To obtain a description of integral neuron processes we used *modulation interference microscopy* (MIM). This method was found to be sensitive to the processes that are coordinated in time and space that allows to study cooperative phenomena in a cell [38–40]. *Interference microscopy* is based on the measurement of the optical path difference between the beam transmitted through the object and the reference beam [41–43] (Figure 3).

The measured value is normalised by the wavelength in order to obtain the phase height of the object at a point:

$$\Phi = \frac{\phi_0 - \phi_{\text{obj}}}{2\pi} \frac{\lambda}{2} - \Phi_0, \quad (1)$$

where ϕ_0 , λ are the initial phase and the wavelength of laser beam, respectively, ϕ_{obj} the phase shift of the object, and Φ_0 the constant phase shift, determined by the choice of phase reference point.

For a heterogeneous object the phase height is:

$$\Phi(x, y) = \int_0^Z (n_{\text{obj}}(x, y, z) - n_s) dz - \Phi_0. \quad (2)$$

Here n_s is a constant refractive index of physiological saline, and $n_{\text{obj}}(x, y, z)$ the cell refractive index in a point with distance z from the mirror layer with the cell. Phase height values at all points of the object form a phase portrait of the cell. Changes of phase height under simultaneously modifications of cell geometry and refractive index are mainly determined by the latter parameter – $n_{\text{obj}}(x, y, z)$. Variations of the phase height provide data on the dynamics of local refractive index originating from regular processes in the plasma membrane and cytoplasm. We used Fourier

analysis to reveal frequencies of these processes. Experiments were done on the modulation interference microscope MIM 2.1 developed by Amphora Laboratories [43]. MIM 2.1 is based on the scheme of the Linnik' interferometer, the wavelength of the laser beam is 532 nm, the power of laser beam transmitted through the cell is 2 mW. To reduce external vibration artifacts, we used a sturdily constructed interferometer block, a two-level vibration isolation system, and a recording channel for the subtraction of low frequency vibration noise. Motoneurons of the pond snail were put into the containment chamber with a mirror bottom layer. The chamber was filled by physiological solution. The working field size was $80 \times 80 \mu\text{m}$ (1024×1024 pix) and scanning rate was 500 pix/s. Data acquisition was performed in a row of points (4, 8 or 16) on a phase portrait. The diameter of a point was approximately 78 nm (1 pix). The sampling interval for individual point in the row was 8, 16 or 32 ms. For each experiment the measurements were done in 6–10 different parts of the neuron. The ultimate concentration of SNP in the chamber was 0.5 mM.

3. Results and Discussion

It was shown that SNP activates voltage-dependent K^+ -channels of neurons. Figure 4 represents patch-clamp recordings of K^+ current through a membrane patch with two channels.

The opening of channels is rare in the control experiments (Figure 4a). In three minutes after SNP addition the duration of the open state increased and the duration of the closed state decreased. Activation of K_V -channels can be seen by the increase in the duration of the state when two channels are opened simultaneously (Figure 4b). The duration of states with one open and two concurrently open channels increased up to the 12th and 14th min (Figure 4c) and after that stabilised. Solution

Figure 4. Patch-clamp recordings of K^+ current through the membrane patch with two voltage-dependent K^+ (K_V)-channels. Scale bar: vertical – current amplitude, 1 pA; horizontal – time, 50 ms. Horizontal lines mark level of zero current, *a* – before the addition of sodium nitroprusside (SNP), *b* – at the 3rd minute after SNP addition, *c* – at the 12th min after SNP addition, *d* – in 10 min after solution exchange. SNP concentration was 0.1 mM.

exchange partly removed the nitric oxide effect, as can be seen by the decrease in the duration of the open state of channels (Figure 4d). Our results are in agreement with the data on SNP effect on the voltage-dependent K^+ -channels of smooth muscle cells [44, 45]. This may be evidence of the common principles of NO influence on similar channels in different cell types. The activation of K^+ -channels may result from the nitrosylation of their thiol groups and formation of disulfide bonds. We suppose that the modification of channel activity also can be caused by changes of lipid–lipid and lipid–protein interactions in the channel's surrounding due to the NO^+ reaction with carboxyl COO^- groups of lipids and proteins [46]. The latter can modify membrane surface charge and the amount of the membrane-bound Ca^{2+} (Ca_{mb}^{2+}). To test whether NO modifies these membrane properties we studied SNP influence on Ca_{mb}^{2+} .

It was shown that NO causes the desorption of Ca^{2+} from the plasma membrane by the 4th min after NO-donor addition into the chamber with neurons (Figure 5, curve a). It occurs a bit later than the beginning of K^+ -channel activation. So, we suppose that NO first activates K^+ -channels, leading to plasma membrane hyperpolarisation. Afterwards NO causes the desorption of Ca_{mb}^{2+} from the plasma membrane and after that the change of the membrane surface charge alters ion channel activity as a feedback regulation [47]. SNP can affect the amount of Ca_{mb}^{2+} through the interaction of NO^+ with negative charged groups on the surface of the plasma membrane and displacement of Ca^{2+} by NO^+ [46]. This effect is non-specific and can be observed for all types of membranes, so we suggested that NO could alter the physico-chemical properties of organelles membranes. To show whether internal membranes participate in the NO-induced response of neurons

Figure 5. Influence of nitric oxide on the amount of bound Ca^{2+} . Sodium nitroprusside was added at the zero point of time. On the X axis – time of neuron's incubation in the SNP solution (1 mM), on the Y axis – intensity of chlortetracycline (CTC) fluorescence. The decrease in the intensity of CTC fluorescence corresponds to the decrease in the amount of bound Ca^{2+} . SNP causes the desorption of Ca^{2+} from the plasma membrane surfaces in 3–4 min after SNP addition (curve a). In 25–26 min after SNP addition NO leads to the desorption of Ca^{2+} from the intracellular membrane surfaces and to the Ca^{2+} exit from its stores (curve b).

we studied NO effect on the $\text{Ca}_{\text{mb}}^{2+}$ amount and the microviscosity of organelle membranes.

Figure 5, curve b represents the dependence of the amount of Ca^{2+} bound on the surface of organelles membranes and in the hydrophobic Ca^{2+} stores. SNP caused the decrease in the amount of intracellular bound Ca^{2+} on the 26th min after SNP addition into the experimental chamber, much later than for plasma membrane. This process had two stages: the first and faster one was similar to the Ca^{2+} desorption from the plasma membrane and the second one was slower. We suppose that the first stage corresponds to the desorption of Ca^{2+} from the surface of intracellular membranes and the second stage is responsible for Ca^{2+} exit from the deeper organelle compartments and Ca^{2+} -binding proteins. The delayed response of intracellular membranes and Ca^{2+} stores is related to the penetration of NO inside the cell and the formation of NO^+ in the cytoplasm. The decrease in the amount of Ca^{2+} bound on the surface of organelle membranes may result from the direct interaction of NO^+ with negatively charged Ca^{2+} -binding groups of the membrane lipids and proteins. The exit of Ca^{2+} from the deeper organelle compartments and Ca^{2+} -binding proteins can be caused by the evoked reactions inside the cell and brings to the increase in the amount of free cytoplasmic Ca^{2+} . Our results are in accordance with data on NO^+ -induced inactivation of ER Ca^{2+} -ATPases, depletion of ER Ca^{2+} stores and Ca^{2+} exit from mitochondria [21–23].

By resonance Raman spectroscopy it was shown that SNP affects the microviscosity of the cytosome membrane. The ratio I_{1526}/I_{1160} decreased in 25 min after SNP addition into the chamber with neurons (Figure 6), corresponding to the decrease in the cytosome membrane microviscosity. NO effect was reversible and approximately in an hour membrane microviscosity increased to its control level. In [48] it was shown that NO did not react with the fatty acids of lipids but interacted with lipid radicals that appeared during membrane oxidation. So, we suggest that the change of cytosome membrane microviscosity is not caused by direct reaction of NO with membrane lipids but is related to the NO-induced processes in the cytoplasm and plasma membrane. One of the supposed functions of cytosomes is

Figure 6. Nitric oxide effect on the microviscosity of the cytosome membrane. On the X axis – time after addition of sodium nitroprusside (0.5 mM) to the neurons, on the Y axis – meaning of I_{1526}/I_{1160} ratio. The decrease in this ratio corresponds to the decrease in the membrane microviscosity. 0.95% confidence interval is indicated by error bars. * $p < 0.05$ vs. control.

Figure 7. Optic photograph (a) and the phase portrait (b) of neurones of the pond snail *L. stagnalis*. Axis on photo b: X, Y – sizes of the visible field, 1024×1024 pixels ($80 \times 80 \mu\text{m}$), Z – the phase height, nm.

a reversible accumulation and storage of Ca^{2+} ions [31]. So, the redistribution of bound and free cytoplasmic Ca^{2+} can influence the cytosomes' function and alter microviscosity of their membranes. Probably, nitric oxide, via processes in the plasma membrane and cytoplasm, can also influence the membrane microviscosity of other organelles, and thus modulates the activity of membrane-bound enzymes and transmembrane proteins.

So, NO can evoke interrelated processes in the plasma membrane and inside the cell. For the description of the integral NO-induced processes we used interference microscopy. Figure 7(a, b) represent an optic photograph and a phase portrait of *L. stagnalis* neurons, respectively.

The phase profile is determined by the organelles' location, the cytoskeleton structure and plasma membrane protein and lipid domains. The highest points in the phase portrait (white) correspond to the places with the highest refractive index. We revealed that in the rest conditions the phase height of neurons undergoes changes with frequencies in the ranges 0.2–1.5 Hz (Figure 8a).

Figure 8. Nitric oxide alters the refractive index of neurons. On the X axis – frequency of changes of the refractive index, Hz; on the Y axis – the amplitude of these changes, nm. a, c – before the addition of sodium nitroprusside (0.5 mM) to the chamber with neurons, b, d – in 25 min after SNP addition. Changes of the refractive index relate to the regular processes in the plasma membrane and cytoplasm.

In 20 min after SNP application, the amplitudes of all fluctuations increased and new frequencies around 3 Hz and in the region of 17–28 Hz appeared (Figures 8b and d). NO-induced changes of the phase height subsided in an hour. It was shown that group of frequencies 0–2 Hz is related to the reactions that occur in the membranes [39]. It was supposed that cooperative processes of the formation of lipid rafts and changes of the membrane surface charge and membrane potential account for these frequencies. It has been shown that 3 Hz is a frequency of actin filament movements and that vesicles movement has frequencies in the wide range 8–40 Hz [49, 50].

We suppose that the increase in the amplitude of frequencies 0.2–2 Hz and the appearance of the frequencies 3 Hz and 17–23 Hz result from the influence of NO on the protein and phospholipid clusters of the plasma membrane, cytoskeleton filaments and vesicle movement. This hypothesis is confirmed by the data on NO interaction with exocytosis-related proteins and NO-induced activation of the fusion of some exocytotic vesicles [51]. Besides, it is known that NO causes the increase of spontaneous release of neurotransmitter [52], that can result from the influence of NO on the fusion of vesicles with the plasma membrane.

4. Summary and Conclusions

NO evokes different time-scale processes that take place in the plasma membrane and cytoplasm and occur on many levels from the molecular level to the whole cell level.

We have shown that exogenous nitric oxide activates K_V -channels. For the first time it was established that NO causes desorption of Ca^{2+} bound on the plasma membrane and influences membrane properties of intracellular organelles. We observed NO-induced decrease in cytosome membrane microviscosity and a two-stage decrease in the amount of intracellular bound Ca^{2+} . We suppose that the two-stage kinetics of the latter process is related to the (i) desorption of Ca^{2+} ions from the organelles surfaces and (ii) Ca^{2+} exit from the organelles Ca^{2+} -binding stores. Also a new finding is that NO alters such integral cellular property as refractive index. Changes of the neuronal refractive index originate from the cooperative NO-induced processes in the plasma membrane and cytoplasm. We established the characteristic frequencies of these changes in the range 0–30 Hz.

Obtained data are summarised in the following scheme (Figure 9). The first target for exogenous NO is the plasma membrane and its structures. NO first activates K_V -channels which lead to the hyperpolarization of plasma membrane and change of the membrane surface charge. NO interacts with negative-charged membrane groups and causes the desorption of the membrane-bound Ca^{2+} , which in its turn alters transmembrane potential and ion channels activity. Besides, NO affects plasma membrane proteins and phospholipids clusters. It can be both an indirect

Figure 9. Proposed scheme of nitric oxide influence on neurons. NO activates K_v -channels and causes desorption of Ca^{2+} from the surfaces of plasma membrane. NO also affects formation of protein and lipid clusters. Inside the cell NO causes the decrease in the microviscosity (η) of cytosomes membranes, exit of Ca^{2+} from endoplasmic reticulum (ER) and mitochondria and changes the cytoskeleton organization.

effect of NO-induced modification of ion channel activity or a direct NO interaction with protein thiol groups and lipid carboxyl groups and displacement of Ca_{mb}^{2+} by NO^+ . Remarkably, NO leads not only to the changes of plasma membrane properties but also to the modification of the properties of subcellular organelles, which do not directly participate in the signal transduction. NO causes the decrease in the membrane microviscosity of cytosomes, which play the role of Ca^{2+} stores [31]. NO brings to the desorption of Ca^{2+} from the surface of the intracellular membranes and to the exit of Ca^{2+} from the deeper organelle compartments and Ca^{2+} -binding proteins. These processes lead to the increase in the concentration of free cytoplasmic Ca^{2+} , which affects various enzymes, Ca^{2+} -dependent channels and, as a result, the activity of the whole neuron. Redistribution of the bound and cytoplasmic Ca^{2+} influences cytosome function and alters the viscosity of their membrane. Nitric oxide also affects refractive index of neurons. It results from NO-induced changes of cytoskeleton organisation and vesicle movement and interactions of NO with proteins and lipids of the plasma membrane and actin microphylaments. Reorganisation of the cytoskeleton alters neuron shape and compartmentalisation. Importantly, that changes of physico-chemical properties of the cellular membranes and modification of cytoskeleton structure and neuron compartmentalisation are prolonged and thus can be among the mechanisms that provide signal transduction and information encoding.

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