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Research Article

THE EFFECT OF CP-6 ON THE TRANSPORT OF MEDIATORS OF NMDA-RECEPTORS AND Ca2+-CHANNELS IN SYNAPTOSOMES OF RAT BRAIN

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ABSTRACT

L Glutamate activity was judged by the change in the intensity of the fluorescent signal, by the change in the cytoplasmic levels of free calcium [Ca2+]in. The study was conducted according to the Weiler method. Synaptosomes were isolated from rat brain using two-stage centrifugation. The entire isolation procedure was carried out at 4°C. To measure the amount of cytosolic Ca2+ synaptosomes were calculated according to the Grinkevich equation. An increase in the concentration of [Ca2+]in caused by L glutamate, primarily due to the activation of membrane permeability, the movement of Ca2+ into the cell, and the release of Ca2+ from intracellular depots. CP-6 competes with L glutamate for the glutamate binding site of NMDA-receptors. L Glutamate partially reduces the effect of CP-6, which may indicate that part of the external calcium also comes under the influence of CP-6 through the open glutamate binding site and in place calcium channels of the NMDA-receptors. It was revealed that CP-6 increases fluorescence and the level of [Ca2+]in, respectively, in synaptic membranes compared to the control. This data gives, about a possible competition between CP-6 and L glutamate for the site of regulation of the opening of ion channels of NMDA-receptors.

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KEYWORDS

NMDA-receptors, synaptosomes, L glutamate, CP-6.

INTRODUCTION

Intracellular free Ca2+ concentration widely varies depending on its location. The cytoplasmic [Ca2+] ([Ca2+]c) under resting conditions is ~10-7M, 104 times lower than [Ca2+] in the extracellular millieu (~10-3M). Inside the cell, Ca2+ levels in the nuclear matrix ([Ca2+]n) and in the mitochondrial matrix ([Ca2+]mt) are similar to that in the cytoplasm. However, other intracellular organelles, known as Ca2+ stores, can accumulate Ca2+ and maintain a higher [Ca2+] than the cytoplasm (1-5×10-4M). The main internal Ca2+ store is the endoplasmic reticulum (ER), and in muscle cells, the sarcoplasmic reticulum [1,2]. The low [Ca2+]c is maintained through the action of the plasma membrane Ca2+ transport ATPase (PMCA) and Na+/Ca2+ exchanger (NCX) in a resting cell. Upon elevated [Ca2+]c, this activity is complemented by the sarcoendoplasmic reticulum Ca2+-ATPase (SERCA) that fills the ER/SR Ca2+ store and to a lesser extent, by the mitochondrial Ca2+ uniporter (mtCU). All these proteins sense and are activated by Ca2+, and therefore any elevations in [Ca2+]c stimulate removal of cytoplasmic Ca2+, resulting in a homeostatic control of [Ca2+]c. Nevertheless, various cell stimuli such as membrane depolarization, extracellular signaling molecules, or intracellular messengers, promote an increase of [Ca2+]c from 100nM to 1 μM or more. This increase results from either the influx of extracellular Ca2+ via the plasma membrane (PM) Ca2+ channels or the release of Ca2+ from internal stores mostly via the 1,4,5-triphosphate receptor (IP3R) and ryanodine receptor (RyR) from ER/SR. The [Ca2+]c increase is usually steep, followed by a decay giving rise to [Ca2+]c spikes or repetitive [Ca2+]c oscillations, which are supported by multiple positive and negative feedback effects of Ca2+ favoring synchronized activation and rapid deactivation of the Ca2+ channels and by the homeostatic regulation of the Ca2+ removal mechanisms. The Ca2+-regulated proteins present different thresholds for activity depending on their function. For example, PMCA and SERCA pumps have high affinities for Ca2+ and low pumping rate (≈30 and ≈10 Hz, respectively) [3,4], which make them suitable to respond to modest elevations in cytoplasmic Ca2+ levels and to reestablish the resting Ca2+ level. NCX and MCU, show a lower affinity for Ca2+ and greater transport rates (150 - 300 Hz for NCX, [5]) and thus can limit larger [Ca2+]c transients. Each cell type presents a unique combination of Ca2+ channels and pumps to create a cell type-and agonist-specific calcium signal that suits their physiological requirements [6-8].

The concentration of intracellular Ca2+ in neurons is a homeostatic parameter and under physiological conditions the transmembrane calcium exchange is regulated by several mechanisms. On the one hand, Ca2+ concentration increases as a result of the discovery of ligand-controlled and potential-controlled calcium channels, and the release of Ca2+ bound by intracellular depots upon activation of IP3 or ryanodine receptors of the endoplasmic reticulum. On the other hand, the excess concentration of intracellular Ca2+ is counteracted by ATP-dependent mechanisms of Ca2+ "pumping" through the plasmolemma and sequestration in the endoplasmic reticulum, Ca2+/Na+ transmembrane exchange and other buffer and / or Ca2+-binding processes. Coordinated management of these mechanisms controls the level of [Ca2+]in, allowing it to fluctuate within certain limits and with a

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certain spatio-temporal pattern to provide a variety of Ca2+-dependent processes of intracellular signal transduction.

Brain functions are manifested at specific synapses through release of neurotransmitters inducing a number of biochemical signaling events in postsynaptic neurons. One of the most prominent of these events is a rapid and transient rise in calcium levels. This local increase in calcium concentrations results in a number of short-term and long-term synapse-specific alterations. These include the insertion or removal of specific calcium channel subunits at or from the membrane and the post-translational modification or degradation of synaptic proteins [7;8;9].

The violation of calcium homeostasis in nerve cells is accompanied by many brain diseases. For example, in cerebral ischemic strokes, an avalanche-like increase in the concentration of calcium in the cytoplasm of neurons plays a major role in the chain of pathological disorders that lead to cell death by apoptosis, which causes all processes occurring in ischemic brain tissue to be termed the "calcium hypothesis of ischemic cell death".

With calcium deficiency, the release of the neurotransmitter is blocked, the excitation and inhibition mechanisms are violated.

L glutamate in neurons can develop neurodegenerative processes associated with violation of Ca2+ regulation, which trigger intracellular signaling cascades leading to the death of neurons [10]. It is known that the neurotoxicity of L glutamate is involved in the pathogenesis of such socially important neurological diseases as epilepsy, ischemic stroke, migraine, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease. In this regard, the study of the mechanisms of neurotoxic action of L glutamate and agonists of its receptors is one of the most topical directions in modern neuroscience.

To date, the important role of the adrenaline, serotonin, and acetylcholine receptors in modulating the functional interactions of neurons has been convincingly established. Particular attention is paid to the presynaptic glutamate receptors, which, according to numerous published data, play an important role in modulating both excitatory and inhibitory neurotransmission, thereby regulating the activity of brain neural networks [11].

Nevertheless, despite numerous studies, ideas about the role of glutamate receptors in modulating inhibitory neurotransmission are very ambiguous. It has been reported that activation as kainate [12-15] leads to increased GABA-ergic neurotransmission. At the same time, several data are indicating the inhibition of GABA neurosecretion under the action of glutamate receptor agonists [16-19]. What may be the mechanisms for the realization of such multidirectional effects resulting from the activation of the same ionotropic receptors remains unclear.

It should be noted that the vast majority of data on the effect of L glutamate on neurotransmission processes were obtained in electrophysiological experiments in which the main criterion for evaluating the effect of activation of presynaptic L glutamate receptors was the change in the frequency and amplitude of the registered synaptic currents in postsynaptic structures. The extremely small geometric dimensions of most nerve terminals are a serious obstacle to the successful conduct of direct measurements of the corresponding phenomena in presynaptic formations. In this regard, information on those intracellular processes developing in the presynaptic nerve structures was carried out using fluorescent probes.

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Ca2+is a ubiquitous intracellular messenger that controls diverse cellular functions but can become toxic and cause cell death. Selective control of specific targets depends on spatio-temporal patterning of the calcium signal and decoding it by multiple, tunable and often strategically positioned Ca2+sensing elements. Ca2+ is detected by specialized motifs on proteins, which have been biochemically characterized decades ago. However, the field of Ca2+sensing has been reenergized by recent progress in fluorescent technology. These approaches exposed local Ca2+ sensing mechanisms inside organelles and at the organellar interfaces, revealed how Ca2+ binding might work to open some channels, and identified disorders linked to a variety of Ca2+ sensing proteins. We here, attempt to place these new developments in the context of intracellular calcium homeostasis and signaling [17].

The operation of Ca2+-signalling systems is constantly under review by an internal quality-assessment mechanism that can respond to changes in the properties of its output signal. We propose the hypothesis that Ca2+ itself has an important function in this internal assessment mechanism by remodelling its own signalling pathway. Several important disease states (hypertension, heart disease, diabetes, manic depression, Alzheimer's disease) might result from abnormal remodelling of Ca2+ signalling.

The study of the mechanisms of calcium homeostasis regulation in excitable cells, the search for biologically active substances and physical factors that affect this homeostasis is one of the most urgent tasks of modern science.

Based on this, our goal is to study the effect of CP-6 on the mediator transport of glutamate-binding NMDA-receptors in rat brain synaptosomes.

Material and methods

Experiments were conducted on 20 outbred male albino rats weighing (200-250 g) contained in a standard vivarium ration. All experiments were performed in accordance with the requirements of "the World Society for the Protection of Animals" and "European Convention for the protection of experimental animals" [20]. Synaptosomes isolated from rat brain by a two-step centrifugation [21]. The whole procedure of selection was carried out at 4°C. To measure the amount of cytosolic Ca2+ was calculated from the equation of Grinkevich [22] in synaptosomes isolated from brain of rats placed in an environment similar to, the one that was used to isolate cells were added 20 μM of chlortetracycline (CTC). Incubated for 60 min to achieve maximal interaction with the membrane -CTC Ca2+ as in plasma, and intracellular membranes. CTC excitation wavelength - 405 nm, recording - 530 nm. Results are expressed as a percentage, taking 100% of the difference between the maximum value of fluorescence intensity (fluorescence dye, a saturated Ca2+) and its minimum value (in the absence of fluorescence of the indicator of Ca2+) obtained after adding ethylene-glycol-bis-amino-ethyl-tetra-acetate EGTA.

Statistical analysis

The measurements were made using a universal spectrometer (USB-2000). Statistical significance of differences between control and experimental values determined for a number of data using a paired t-test, where the control and the experimental values are taken together, and unpaired t-test, if they are taken separately. The value of P <0.05 indicated a statistically significant differences. The results obtained are statistically processed to Origin 7,5 (Origin Lab Corporation, USA).

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Results and discussion

Investigation of the effect of L glutamate on the level of cytoplasmic calcium in brain synaptosomes of rats.

Synaptosomes obtained from rat brain were used in the work, which is an adequate and convenient model for studying presynaptic processes. The activity of L glutamate was judged by the change in the intensity of the fluorescent signal, by the change in the cytoplasmic levels of free calcium [Ca2 +]in.

A fluorescence ratio excited by light at 340 and 380 nm (F340/F380) in synaptosomes was established with the help of the Ca2+ -sensory chlortetracycline probe (CTC). When Ca2+ was removed from the extracellular medium, preincubation of EGTA resulted in a 10% decrease in fluorescence. In the presence of EGTA in the incubation medium (Fig.1), L glutamate in concentrations of (10-100 μM) dose-dependently increases the level of fluorescence by 25-43%, which indicates an increase in [Ca2+]in concentration caused by L glutamate, primarily due to activation of membrane permeability, displacement of Ca2+ into the cell and release of Ca2+ from intracellular depots (Fig.2).

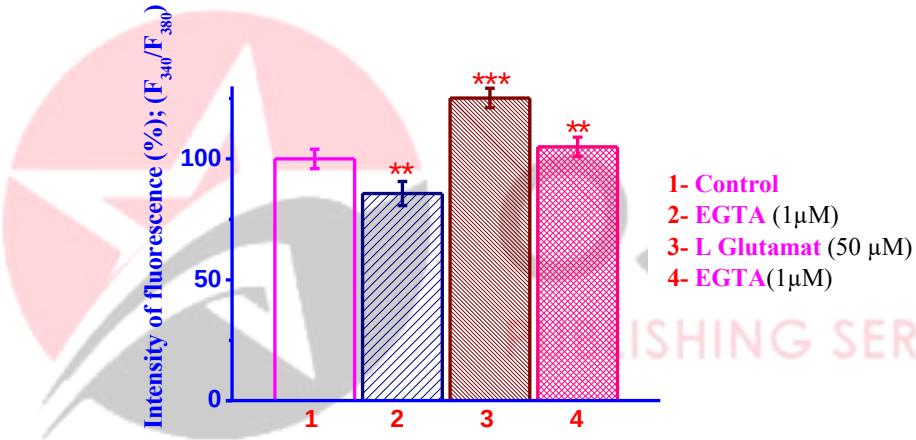

Fig.1. Fluorescence intensity change with L glutamat (10-50 μM) when incubated with rats of brain tumor synaptosomes EGTA (1 mM). Increased fluorescence intensity induced by L glutamat (50 μM). Reliability level * - P <0,05; ** - P <0.01; * - P <0.001. (n = 6).**

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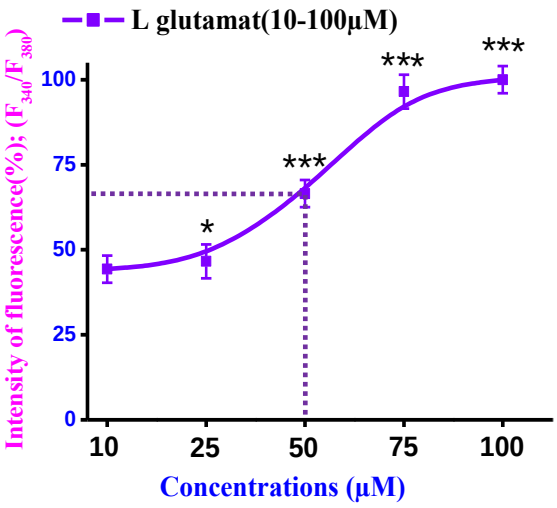

Fig.2. The dose-dependent effect of L glutamate on the level of [Ca2 +]in the brain synaptosomes of rats. Reliability level * - P <0,05; ** - P <0.01; * - P <0.001. (n = 6).**

In addition to increasing the level of [Ca2 +]in due to entry from outside the cell, the processes of maintaining its high concentration in the cytosol due to the release of calcium from the membranes of the endoplasmic reticulum and mitochondria, as well as the disturbance of the processes of its sequestration, are of great importance.

It is known that the change in calcium transport by presynaptic membranes is accompanied by an increase in glutamatergic transmission, which is due to an increase in the release of L glutamate. Excitatory neurotransmitter L glutamate can cause damage and death of DA neurons, and therefore the damaging effect of glutamate on neurons is indicated by the term "toxicity of excitatory amino acids", or “excitotoxicity”.

The L glutamate excitotoxicity is mediated by NMDA-receptors, named for a specific N-methyl-D-aspartate antagonist. When the L glutamate interacts with these receptors, the ion channels of the neuronal membrane open and the L glutamate enters the neuron. The extensive binding of L glutamate with NMDA-receptors leads to an increase in the current of Ca2+ to the neuron through NMDA-receptor channels. Due to the fact that Ca2+ current amplification is one of the leading mechanisms of neuron death, it can be assumed that the mechanism of excitotoxicity of L glutamate in Parkinson's disease is associated with a massive entrance of Ca2+ into DA-neurons of a black substance. The violation of glutamatergic transmission is now also considered as a leading factor in the pathogenesis of diseases such as epilepsy, Alzheimer's disease, etc. [23; 24; 25; 26].

After that, we conducted experiments effect of CP-6 isolated from the plant Scots pine (Pinus sylvestris L) on the glutamatergic neurotransmitter system and changes [Ca2+]in synaptosomes in rat’s brain.

Preincubation of CP-6 (10-100 µM) with the complex of the CTC-synaptosomes increases the fluorescence and

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accordingly, the level of [Ca2+]in difference from L glutamate (Fig. 4).

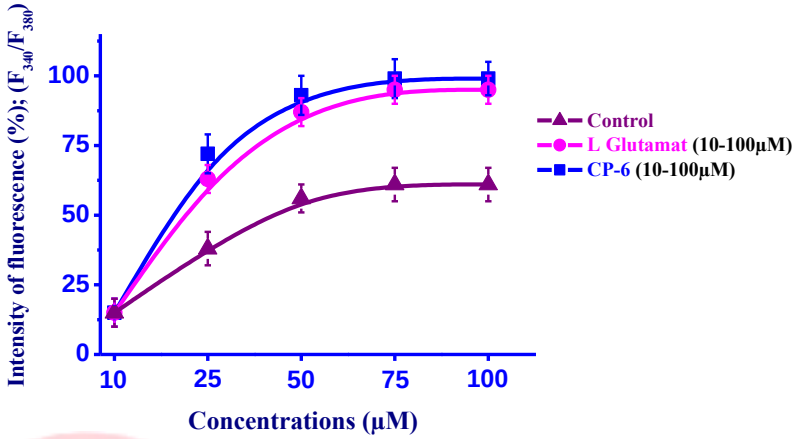

Fig.4. Effects of SP-6e and L glutamate in concentrations (10-100 μM) on the intensity of CTC fluorescence of brain synaptosomes suspension in rats. Reliability level * - P <0,05; ** - P <0.01; * - P <0.001. (n = 6).**

CP-6 (50 μM) reduced the fluorescence and accordingly the level of [Ca2+]in against the background of L glutamate (50 μM) on the complex of CTC-synaptosomes (Fig.5).

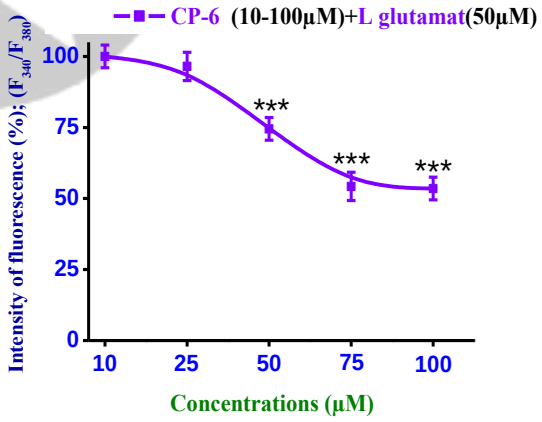

Fig.5. Effect on fluorescence intensity in synaptosomes suspension in conditions of incubation with CP-6 (10-100 μM) L glutamate (50 μM). Reliability level * - P <0,05; ** - P <0.01; * - P <0.001. (n = 6).**

The preliminary preincubation of CP-6 (10 μM) with synaptic membranes, then the addition of CTC- L glutamate resulted in a decrease in fluorescence and a level of [Ca2+]in, respectively. A dose-dependent increase in CP-6 concentration to (10-100 μM),

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respectively, resulted in a dose-dependent decrease in the effect of L glutamate (Fig. 5).

The effect of L glutamate was observed depolarization of the synaptic membrane and an increase in intracellular calcium without an appreciable change in the concentration of internal sodium ions. Increase in synaptosomal calcium was inhibited by the addition of L glutamate. Activation of L glutamate receptors causes the opening of calcium channels ionotropic receptors, calcium influx into synaptosomes and depolarization of the synaptosomal plasma membrane, followed by the release of amino acid neurotransmitters. L Glutamate partially reduces the action of CP-6, which may indicate that part of the external calcium comes under the influence of SP-6e also through the open glutamine site and in place of calcium channels NMDA-receptors. Even the preliminary addition of L glutamate does not completely abolish the action of CP-6, which may indicate that CP-6 has several mechanisms of action on rat brain neurons, the result of which is an increase in [Ca2+]in.

From the literature data it is known that, Mg2+ ions selectively block the activity of NMDA-receptors. Glycine enhances NMDA-receptor responses by increasing the frequency of channel opening. In the complete absence of glycine, the receptor is not activated by L glutamate.

Indeed, the addition of glycine to the incubation medium (5 µM) enhanced the L glutamate-dependent increase in fluorescence by 15-22%. At the same time, Mg2+ ions (50 µM) inhibited L glutamate-induced Ca2+ release from intracellular depots (Fig.6).

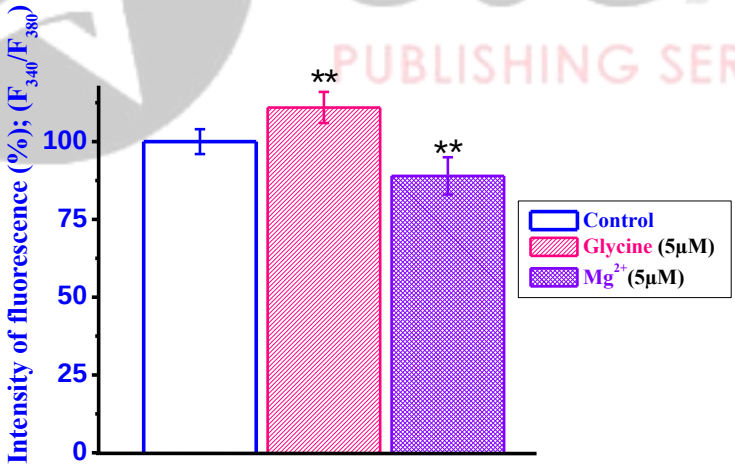

Fig.6. Effect of glycine and Mg2+ ions on L glutamate-inducible Ca2+ intracellular depot. Reliability level * - P <0,05; ** - P <0.01; * - P <0.001. (n = 5).**

It is known that glycine stimulating effects of L glutamate and competitive receptor antagonists such as AP5, AV-2-1 toxin can prevent activation of L glutamate. Other drugs and Mg2+ ions may block the open channel through the non-competitive

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antagonism. These medications include experimental neuroprotective drug MK-801 and argiolobatin [27-34].

In order to identify, possible interaction with polyphenol SP-6 areas over stimulation NMDA-receptor responsible for the opening of calcium channels, investigated its effect on the background of the non-competitive antagonists such as magnesium ions, argiolobatin and calcium channel blockers - nifedipine.

It is shown that magnesium ions in millimolar concentrations significantly inhibit the fluorescence of the L glutamate-CTC-synaptosomes complex. The inhibitory effect of magnesium ions against the background of CP-6 (50 µM) of the fluorescence of the CTC-synaptosomes complex did not change.

In these studies, it was shown that in the presence of CP-6, the inhibitory effect of magnesium ions (50 µM) was not observed. This is probably due to the fact that there is no competition between Mg2+ and CP-6 over sites that stimulate the opening of ion channels. It has also been shown that the action of argiolobatin (10 µM) on the calcium channels of the NMDA-receptor in the presence of CP-6 (50 µM) does not change (Figure 7).

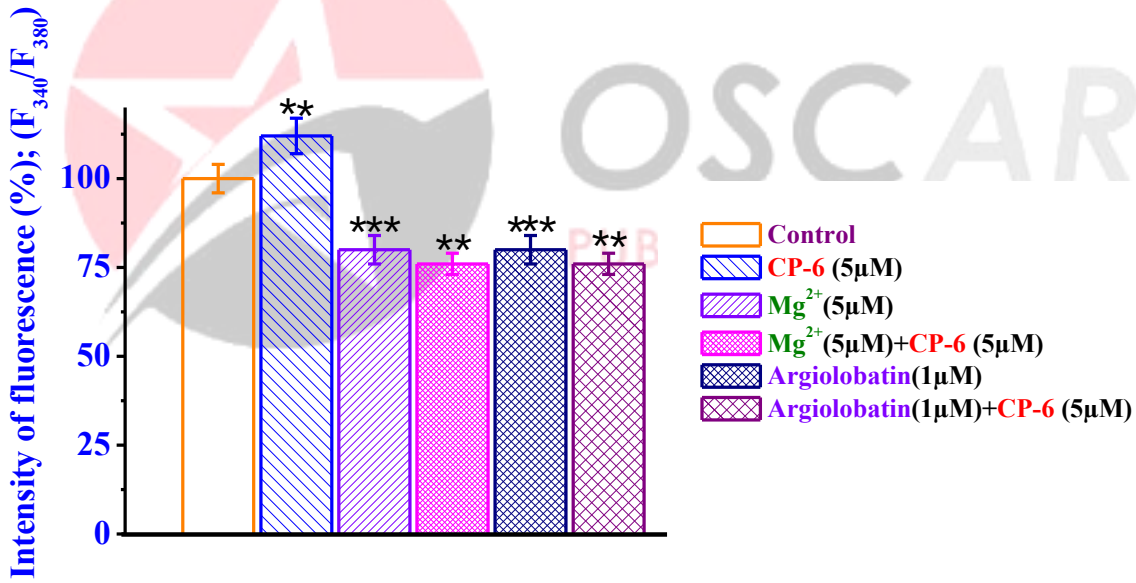

Fig.7. Effect of non-competitive NMDA-receptor antagonists Mg2+ and argiolobatin on the background of CP-6 on fluorescence intensity and the level of [Ca2+]in in the brain synaptosomes of rats. Reliability level * - P <0,05; ** - P <0.01; * - P <0.001. (n = 6).**

When investigating the effect of SP-6 on calcium-dependent NMDA-receptor processes were studied against the background of the blocker of the L-type Ca2+ channels of nifedipine in the brain synaptosomes of rats.

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Preincubation of nifedipine (0.01 µM) with the suspension complex of the CTC-synaptosomes resulted in a decrease in fluorescence. Preincubation of CP-6 (50 µM) with the suspension complex of the CTC-synaptosomes, no decrease in fluorescence. Preincubation of SP-6 (50 µM) against a background of nifedipine (0.01 µM) with a complex of CTC-synaptosomes did not result in a change in fluorescence (Fig.5), indicating that there is no competition between SP-6 and nifedipine for the site of regulation of dihydropyridine-sensitive calcium channels.

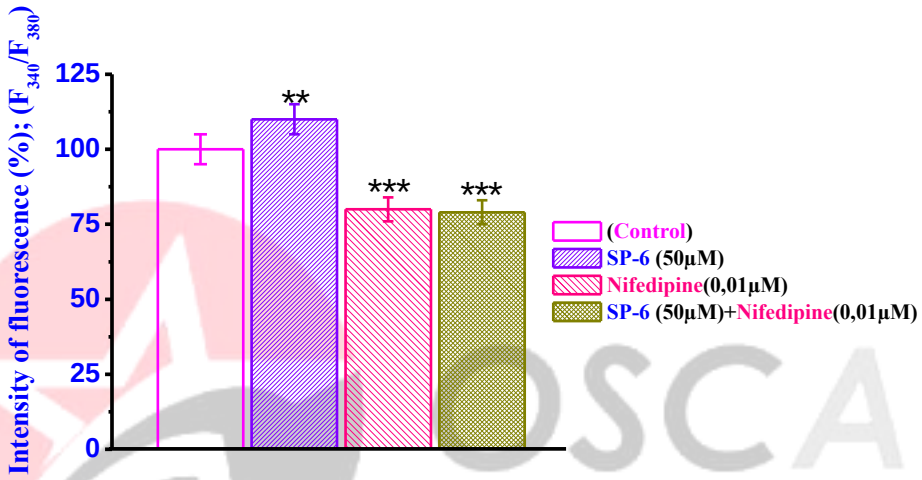

Fig.5. Effect of CP-6 on calcium-dependent NMDA-receptor processes on the background of nifedipine. Reliability level * - P <0,05; ** - P <0.01; * - P <0.001. (n = 6).**

This is explained by the fact that; CP-6 does not work for the site of regulation of the dihydropyridine-sensitive calcium channels of the rat brain synaptosomes membrane.

There are numerous Ca2+-signalling systems that are designed to regulate many different cellular processes. This versatility is achieved by the existence of an extensive Ca2+-signalling toolkit that is used to assemble these cell-specific signalsomes that can deliver Ca2+ signals with the spatial and temporal characteristics that are necessary for its many control functions. A major challenge for the future is to determine the differential transcription and expression mechanisms that are responsible for putting together these different signalling pathways. In addition, it will be important to establish the quality-assessment mechanisms that are responsible for maintaining the integrity of these signalling pathways. There is already some indication that Ca2+ itself might function in regulating the stability of its signalling pathways. This Ca2+-dependent quality-assessment mechanism warrants further attention, because there are indications that several pathologies, such as neurodegenerative disease, might develop after the abnormal remodelling of Ca2+ signalling.

CONCLUSIONS

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In these studies, it was found that CP-6 increases the fluorescence and the level of [Ca2+]in, respectively, in the synaptic membranes compared with the control. The results obtained indicate a possible competition between CP-6 and L glutamate for the site of regulation of the opening of ion channels of NMDA-receptors.

It was found that the effect of CP-6 responsible for the opening of calcium channels with other sites of NMDA-receptors against the background of magnesium ions, argiolobatin and nifedipine, a change in the level of [Ca2+]in synaptosomes was not observed.

The results indicate the possibility of using CP-6, as an exciting neurotransmitter in neurodegenerative diseases.

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