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Action of Cytisinum on the Transport Mediators and Calcium Channel of Glutamatergic Neurotransmitter Systems of the NMDA Receptor

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Abstract

Background: The purpose of this study was the effect of the alkaloid Cytisinum regulation of the calcium channels of brain synaptosomes in rats. This enables the regulation of the transport of mediator's glutamatergic neurotransmitter receptors.

Methods: The study was carried out using the Weilers method. Synaptosomes were isolated from the brain of rats by a two-step centrifugation method. The entire isolation procedure was carried out at 4°C. To measure the amount of cytosolic Ca^{2+} synaptosomes were calculated by the Grinkevich equation.

Results: Increase in the concentration of Ca^{2+} ($[Ca^{2+}]i$) caused by glutamate, primarily due to activation of membrane permeability, movement of Ca^{2+} into the cell and release of Ca^{2+} from intracellular stores. Cytisinum does not compete with glutamate for the binding site. Perhaps the effect of Cytisinum is due to the interaction with the ion channels of NMDA receptors. Cytisinum is able to interact with the glutamate binding site of the NMDA receptor and allosteric modulating sites located on the membrane of the ion channels. In these studies it was shown that in the presence of Cytisinum, the inhibitory effect of magnesium ions (10 μM) is not observed. This is probably due to competition between Mg^{2+} and Cytisinum over sites that stimulate the opening of ion channels. Perhaps there is a competition between Cytisinum and nifedipine for the site of regulation of dihydropyridine-sensitive calcium channels.

Conclusion: The action of the alkaloid of Cytisinum is due to the interaction with the ion channels of NMDA receptors. Cytisinum is able to interact with the glutamate binding site of the NMDA receptor and the allosteric modulating sites. There is competition between Mg^{2+} and Cytisinum over sites that stimulate the opening of ion channels and in nifedipine for the site of regulation of dihydropyridine-sensitive calcium channels.

Keywords: synaptosomes, NMDA, glutamate, Cytisinum, nifedipine, Mg^{2+}.

1. Introduction

NMDA receptors (NMDARs) are glutamate-gated cation channels with high calcium permeability that play important roles in many aspects of the biology of higher organisms. They are

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critical for the development of the central nervous system (CNS), generation of rhythms for breathing and locomotion, and the processes underlying learning, memory, and neuroplasticity. Consequently, abnormal expression levels and altered NMDAR function have been implicated in numerous neurological disorders and pathological conditions. NMDAR hypofunction can result in cognitive defects, whereas overstimulation causes excitotoxicity and subsequent neurodegeneration. Therefore, NMDARs are important therapeutic targets for many CNS disorders (Kemp et al., 2002; Jansen et al., 2003; Chazot , 2004; Farlow, 2004; Wood,2005; Cai,2006; Missale et al.,2006; Brown et al., 2006) including stroke, hypoxia, ischemia, head trauma, Huntington's, Parkinson's, and Alzheimer's diseases, epilepsy, neuropathic pain, alcoholism, schizophrenia, and mood disorders. To date, drugs targeting NMDARs have had only limited success clinically due to poor efficacy and unacceptable side effects, including hallucinations, catatonia, ataxia, nightmares, and memory deficits.

A detailed understanding of the mechanisms underlying agonist-induced receptor activation would facilitate development of more selective drugs that target specific NMDAR subtypes and alter their function to a well-defined extent. This chapter will investigate the physiological roles NMDARs play in the mammalian nervous system and the molecular and structural basis of NMDAR activation. One of the main questions that will be addressed is how agonist binding results in opening of the NMDAR ion channel. Although the mechanism coupling ligand binding to channel opening remains incompletely understood for NMDARs, we propose that this process suggests promising approaches to drug design (Antonius et al., 2008).

Glutamate is the major excitatory neurotransmitter, in the mammalian CNS. Not surprising, then, that a large number of physiological functions based on the active use of glutamatergic transmission. It would be impractical to try to describe all the possible physiological effects of the activation of the NMDA-receptor complex. Nevertheless, there are three main physiological functions of NMDA-receptor, which reflect the functional characteristics of these receptors are the basis for the development of pharmacological modulation techniques.

In light of this, special attention attracts glutamatergic system of the brain. The glutamatergic system includes receptors (ionotropic and metabotropic) glutamate and carriers. signal feature in the synapse by means of glutamate is that in addition to neurons in the process are directly involved glial cells: as for the termination of the exciting action of glutamate needed its evacuation from the synaptic cleft, glutamate is transferred into astrocytes, where "neutralized", turning into glutamine (Cycle glutamate/glutamine works much more intense, "reuptake" torus neurotransmitters glutamate neural carriers).

In the pathology, Nicholson et al (Nicholson et al., 1977) showed that hypoxia of the brain tissue causes a rapid increase in the concentration of intracellular calcium in them, the "calcium hypothesis" was transferred to brain neurons to explain calcium-mediated neuronal death in ischemia/hypoxia, hypoglycemia, and epilepsy (Siesjo, 1994). And after the culture of neurons (Rothman et al., 1987) and brain sections (Garthwaite et al., 1986) showed that glutamate and the corresponding excitatory amino acids cause neuronal cell death, the name "excitatory glutamate toxicity" arose. Currently, the hypothesis of "glutamate excitotoxicity" in most cases is considered within the "calcium hypothesis of ischemic cell death" (Kristian et al., 1998). Its main provisions in the application to the tissues of the brain are as follows.

The concentration of free Ca^{2+} in the cytoplasm of cells is about 10-7 M, while in the intercellular environment and in the cellular organelles it is about 10-3 M. As a result of ATP deficiency, the activity of active calcium transporters (Ca-ATPase and Na / Ca-exchangers) decreases The Ca^{2+} concentration in the cytoplasm increases. In addition, the deficiency of ATP leads to a decrease in the membrane potential, which in turn activates the potential-dependent Ca channels, and this leads to an even greater increase in the intracellular Ca^{2+}concentration.

Because of the specific nature of nerve cells, a fatal role in them is played by an increase in the concentration of Ca^{2+} in the presynapses region, which leads to a massive release of various neurotransmitters, among which the exciting mediator glutamic acid has a special role. This is due to the fact that, first of all, the most synapses in the brain (about 40%) are glutamate-ergic and, secondly, the majority of postsynaptic glutamate receptors (NMDA- and AMPA-receptors) control the calcium channels and, correspondingly, even more Increase the concentration of intracellular Ca^{2+}. In connection with this positive feedback, the concentration of cytoplasmic Ca^{2+} in glutamate-sensitive neurons increases dramatically. Therefore, up to now, one more name of the processes

occurring in the ischemic focus of the brain continues to exist: "exciting toxicity" or otherwise "glutamate excitotoxicity" (Kristian et al., 1998; Fohr et al., 1995; Berridge, 1993).

Like other divalent cations Ca^{2+} have a negative modulatory influence on the activity of NMDA-receptor complex. High extra- and intracellular Ca^{2+} concentration reduces the NMDA-receptor channel conductivity (McBain et al., 1994), which can be seen as paradoxical, but very reasonable self-defense mechanism of the neuron from overstimulation. The inhibitory effect of Ca^{2+} could be due to redistribution of the surface charge of the membrane with a specific blocking action ("blockage of the channel"), activation of tyrosine and serine phosphatases that dephosphorylate C-terminal fragments of subunits NR_1 and NR_2, activation of calmodulin, NO-synthetase, as well as depolymerization of actin.

The modulating effect of Ca^{2+} may be mediated by changes in the cytoskeleton. For example, it was found that the activity of NMDA-receptor complex aktinopodobnym regulated protein that is depolymerized by increasing intracellular calcium concentration (Rosenmund et al., 1993) It is believed that the Ca^{2+}-dependent inactivation of NMDA-receptor complex occurs with the participation of several regulatory proteins - α-actinin-2 neyrofilament- a (Ehlers et al., 1998), as well as postsynaptic PSD family of proteins that bind to the subunits NR2 (Harris et al., 1997). The meaning of this regulation is that the NMDA-receptor (more precisely, its channel) is active only when the regulatory proteins bind subunits klasterizuya receptor complex in the synaptic region in close proximity to second messenger systems Clustering is another highly efficient mechanism of self-regulation activity NMDA-receptor complex, since the entrance of Ca^{2+} through the channel associated with the NMDA-receptor, promotes inactivation of receptors due to activation of Ca^{2+} -dependent phosphatase, calmodulin, which prevent binding of NMDA-receptor complex (NR_1 subunit) with elements of the cytoskeleton (Rosenmund et al., 1993; Ehlers et al., 1998).

Purpose: Actions on glutamatergic neurotransmitter system of the NMDA-receptor.

Tasks:

- Study of Cytisinum effect on binding site agonist (glutamate).
- The study of Cytisinum acts on the glycine co-agonist binding site.
- Study on the action of Cytisinum binding site Mg^{2+}.
- The study of Cytisinum binding site of action in the "channel" blockers.

2. 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" (European Convention..., 1986). Synaptosomes isolated from rat brain by a two-step centrifugation (Weiler et al., 1981). The whole procedure of selection was carried out at 4°C. To measure the amount of cytosolic Ca^{2+} was calculated from the equation of Grinkevich (Grynkiewicz et al., 1985) 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 Ca^{2+} 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 Ca^{2+}) and its minimum value (in the absence of fluorescence of the indicator of Ca^{2+}) 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 6.1 (Origin Lab Corporation, USA).

3. Results and discussion

Action alkaloid Cytisinum *(1R, 5S) -1,2,3,4,5,6- hexahydro- 1,5-methano-8H-pyrido [1,2a] [1,5] diazocin-8-one*), isolated from plants (*Thermopsis lanceolata R. Br and Cytisus ruthenicus*) on cytosolic Ca^{2+} in brain synaptosomes of rats.

In the experiments the influence of glutamate on intracellular calcium level was investigated in synaptosomes from rat brain. Pre using the Ca^{2+}-sensitive chlortetracycline (CTC) set ratio of fluorescence excited by light having wavelengths of 340 and 380 nm (F_{340}/F_{380}) in synaptosomes. When removing Ca^{2+} from the extracellular medium, pre-incubation of EGTA resulted in a decrease in fluorescence at 5 %. In the presence of EGTA in the incubation medium, glutamate at concentrations of 1-100 μM dose-dependent increases in fluorescence level at 25-48 %, which indicates an increase in the concentration of cytosolic Ca^{2+} ([Ca^{2+}] i), called glutamate, primarily due to activation of membrane permeability movement of Ca^{2+} into the cell and release of Ca^{2+} from intracellular stores (Fig. 1).

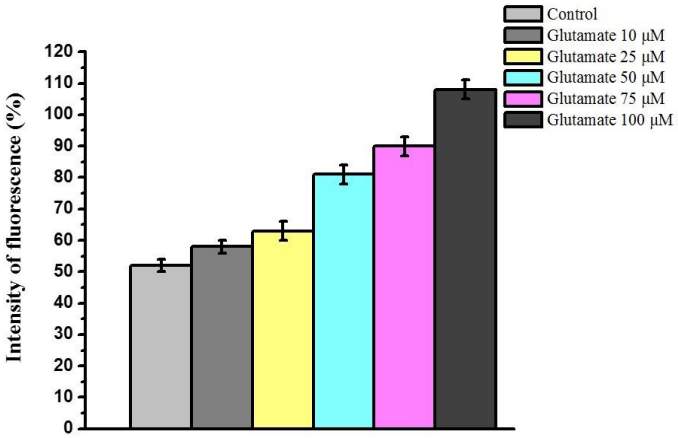

Fig.1. The dose-dependent effect of glutamate on the level of fluorescence.
Reliability index P <0.05

Pre-incubation of the alkaloid Cytisinum reduced fluorescence and therefore the level of cytosolic calcium under the action of glutamate at the complex CTC-synaptosomes (Fig. 2).

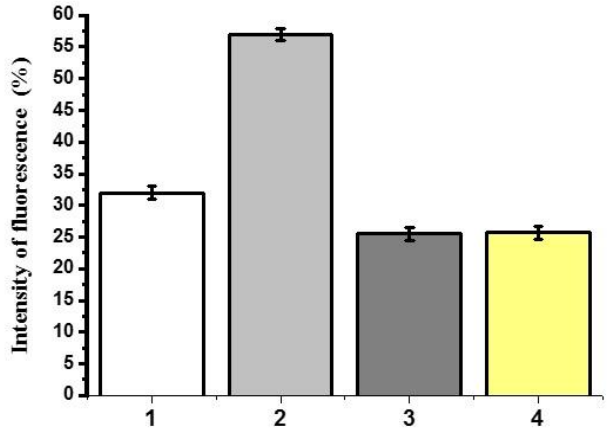

Fig. 2. Influence of the alkaloid Cytisinum in the glutamatergic neurotransmitter system

1 - fluorescence control complex CTC-synaptosomes;
2 - fluorescence complex CTC-synaptosomes by adding 50 μM glutamate;
3 - adding 50 μM glutamate in the background Cytisinum.
4 - Cytisinum 50 μM. Reliability index P <0.05.

In studying the action alkaloid Cytisinum rat brain synaptosomes found that alkaloid Cytisinum significantly reduces the fluorescence, respectively cytosolic calcium levels compared to the control. At the same time increase the concentration of Cytisinum from 10 to 100 μM glutamates for background did not lead to further reduction of the effect of glutamate.

The results show that alkaloid Cytisinum does not compete with the binding site for glutamate. Perhaps the action of Cytisinum has interaction with ion channels of NMDA-receptors.

It is known that, Mg^{2+} ions selectively block the activity of NMDA receptors. Glycine enhances NMDA-receptor responses, increasing the frequency of channel opening. In the complete absence of glycine receptor is not activated by L-glutamate.

Indeed, adding to the incubation medium increased the 5 μM glycine-glutamate-dependent fluorescence increase by 15-20 %. At the same time the ions Mg^{2+} (50 μM) inhibited the glutamate-induced Ca^{2+} release from intracellular stores (Fig. 3).

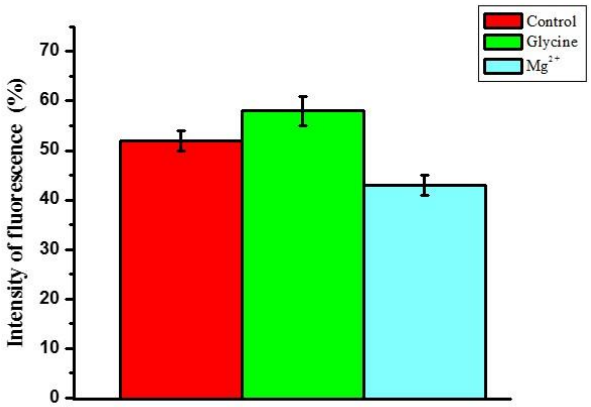

Fig. 3. The action of glycine and Mg^{2+} ions to glutamate-induced intracellular Ca^{2+} stores. Reliability index P <0.05

It is known that glycine stimulating effects of glutamate and competitive receptor antagonists such as AP_5, AV-2-1 toxin can prevent activation of glutamate. Other drugs and Mg^{2+} ions may block the open channel through the non-competitive antagonism. These medications include experimental neuroprotective drug MK-801 and argiolobate (Martin et al., 1977).

In order to identify, possible interaction with Cytisinum areas overstimulation NMDA-receptor responsible for the opening of calcium channels, investigated its effect on the background of the non-competitive antagonists such as magnesium ions, argiolobate and calcium channel blockers – nifedipine.

It is shown that magnesium ions in mill molar concentrations significantly inhibit the fluorescence of the complex glutamate CTC-synaptosomes. The inhibitory effect of magnesium ions fluorescence complex CTC-synaptosomes in the presence of Cytisinum is not changed.

These studies have shown that in the presence of Cytisinum inhibitory effect of magnesium ions (10 μM) was observed. This is probably due to the competition between the Mg^{2+} and Cytisinum for sites that promote opening of ion channels. Also, shown that argiolobate effect on NMDA-receptor calcium channels in the presence of Cytisinum is not changed (Fig. 4).

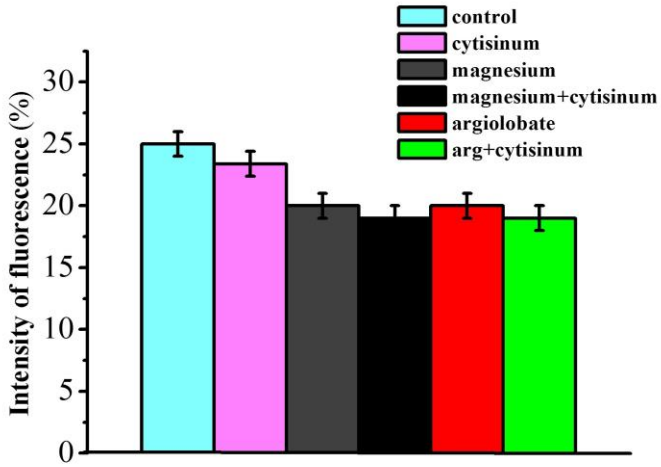

Fig.4. The effect of magnesium ions and Cytisinum at argiolobate fluorescence and cytosolic calcium levels in rat brain synaptosomes. Reliability index P <0.05.

In studying the action of Cytisinum for calcium-dependent processes are NMDA-receptor were examined for background nifedipine (Ca^{2+} channel blocker of L-type) in synaptosomes from rat brain.

Pre-incubation of nifedipine with CTC synaptosomes-complex, led to a decrease in fluorescence. Pre-incubation with Cytisinum CTC synaptosomes-complex, also led to a decrease in fluorescence. Pre-incubation of Cytisinum in the background with nifedipine CTC synaptosomes-complex, led to a slight decrease in fluorescence (Fig. 5), which indicates that competition between Cytisinum and nifedipine for land regulation dihydropyridine-sensitive calcium channels.

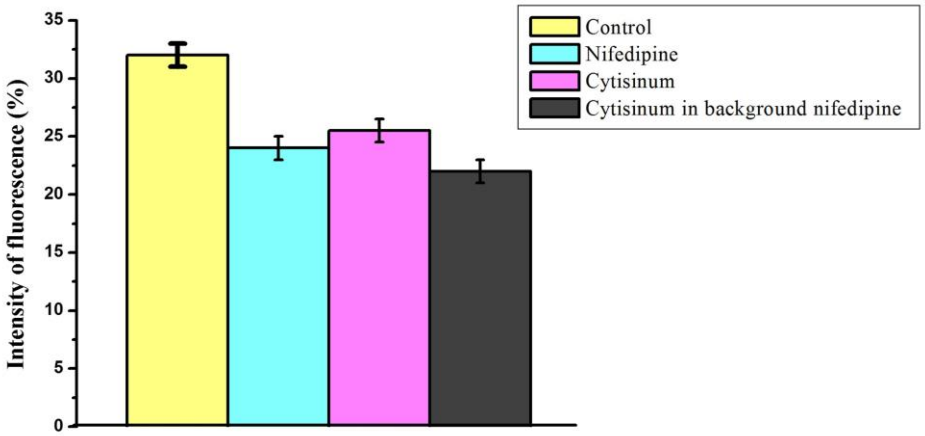

Fig. 5. Effect of Cytisinum for calcium-dependent processes on NMDA-receptor in background nefedipine. Reliability index P <0.05

These results demonstrate the possibility of use in the regulation of Cytisinum dihydropyridine-sensitive calcium channel subtypes major neuronal receptors.

4. Conclusions

Increase in the concentration of cytosolic Ca^{2+} ($[Ca^{2+}]_i$), called glutamate, primarily due to activation of membrane permeability, movement of Ca^{2+} into the cell and release of Ca^{2+} from intracellular stores. Cytisinum does not compete for the glutamate binding site. Perhaps the action of Cytisinum has the interaction with ion channels, NMDA-receptors. The Cytisinum capable of interacting with the glutamate binding site of the NMDA-receptor sites allosterically modulate

disposed on a membrane ion channel. These studies have shown that in the presence of Cytisinum inhibitory effect of magnesium ions (10 μM) was not observed. This is probably due to the competition between the Mg^{2+} and Cytisinum for sites that promote opening of ion channels. There may be competition between Cytisinum and nifedipine for land regulation dihydropyridine-sensitive calcium channels.

As in any field of fundamental science, the completion of one stage does not solve all problems. In what directions can we continue to develop the results and conclusions of this work. First, it is necessary to continue the research of substances for which effects on these channels are found: pharmaceutical preparations and alkaloids. Secondly, it is necessary to continue the search for resonance frequencies for other physiologically significant ions: Na^+, K^+, Cl-, Fe^{2+}, GABA, acetylcholine, etc., and to explore the possibility of simultaneous application of several frequencies to activate various processes in the body.

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