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PLASTICITY OF NEURONAL RESPONSES INDUCED BY LOW CONCENTRATIONS OF EXOGENOUS LIGANDS AFFECTING CELLULAR CALCIUM STORES

Epstein O.I.[1], Zapara T.A.[2], Simonova O.G.[2], Ratushnyak A.S.[2], Shtark M.B.[3]

[1] "Materia Medica Holding" Research and Production Company, Moscow, [2] Technological Institute of Digital Techniques SB RAS, Novosibirsk, [3] Institute of Molecular Biology and Biophysics, Novosibirsk, Russia

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1. ABSTRACT

Modification of reactions caused by repeated influences (plasticity) is a fundamental property of cell. In this study, we have revealed effects of low concentrations of two exogenous modulators of cellular processes (caffeine and cyclosporin A) on neuronal plastic properties. The study was carried out on isolated neurons of Lymnaea stagnalis. It was found that low concentrations of caffeine or cyclosporin A did not cause any changes of the membrane potential (MP) of isolated neurons. However, pretreatment of neurons with low concentrations of caffeine or cyclosporin A reduced the amplitude of MP changes caused by the action of physiological concentrations of these ligands on the average by 30.8% and 29.1%, respectively. The findings permitted implying a significant role of low ligand concentrations in the formation of cell memory and neuronal plastic properties.

2. INTRODUCTION

Lately, evidence of the influence of low concentrations ($10^{-12} \div 10^{-16}$ M) of substances on cellular processes has been accumulated (3,5,10,12,20). In the present study, we attempted to elucidate the effects of low concentrations of two exogenous modulators of cellular processes (caffeine and cyclosporin A) on neuronal plastic properties. Concentrations of $10^{-3} \div 10^{-9}$ M of these substances cause changes of neuronal responses (1,7,14) and are traditionally used in electrophysiological experiments (physiological ligand concentrations).

It is known that targets of caffeine are adenosine receptors of plasma membrane, ryanodine receptors of cellular sarcoplasmic reticulum and mitochondria (1,8,13-14). Adenosine receptor activation inhibits neuronal activity by inducing or modulating ionic currents and reducing transmitter release (6). Adenosine receptors are almost ubiquitous in the brain and affect various K^+ or Ca^{2+} currents through activation of G-proteins (coupled with ion channels, adenylate cyclase or phospholipases) (6). Caffeine acts as a competitive antagonist of adenosine receptors (6). Adenosine inhibits whereas caffeine increases, cell activity. The second targets of caffeine are ryanodine receptors. Binding of caffeine to ranodine receptors facilitates the release of Ca^{2+} from intracellular calcium storage. The process leads to a substantial short-term increase of calcium ion concentration followed by a brief fall of Ca^{2+} below the initial level. The decrease of calcium concentration may be caused by the active calcium-stimulated calcium secretion and subsequent Ca^{2+} reuptake by the calcium storage (1,8,13-14). All these processes lead to changes of the membrane potential and modify cell responses. Cyclosporin A blocks ryanodine receptors, one type of mitochondria pores and cellular phosphatases (7,17,19). We have supposed that pretreatment of neurons with low concentrations of ligands interacting with the receptors controlling the activity of basic signal and energy systems of cells can alter the neuronal response to application of physiological ligand concentrations.

3. MATERIALS AND METHODS

Pond snails, Lymnaea stagnalis (age 0.5-1.5 years), were collected from a wild population in September in ponds and kept during September-December in

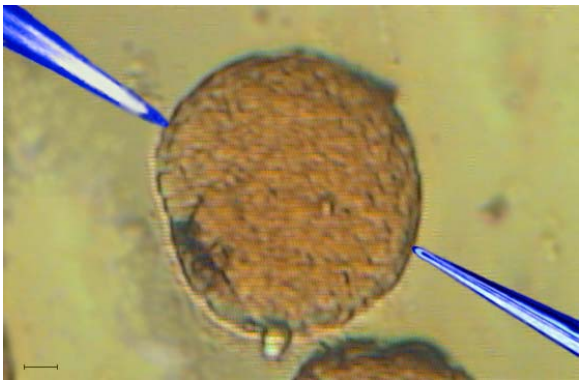

Figure 1. An isolated neuron of Lymnaea stagnalis with microtools: microelectrode for recording membrane potential and micropipette for local application of ligand solutions. There are microelectrode inserted into the neuron (to the left of the cell) and the closely positioned micropipette (to the right of the cell). Scale bar – 5 μm.

laboratory conditions at the temperature of 4 °C in refrigerators in dishes with fresh water in active state. The snails were fed with cabbage and carrots.

Neurons were isolated according to the procedure described by Kostenko *et al.* (11). In briefly, the subesophageal ganglions were transferred to a saline solution containing (mM): NaCl — 30; KCl — 1.6; $CaCl_2$ — 4; $MgCl_2$ — 1.5; $NaHCO_3$ — 10, pH 7.6÷7.8. Isolation of cells was carried out after enzymatic treatment in pronase solution (0.03%, 45-60 min). After digestion, the ganglions were washed 5 min by the saline solution. Mechanical disaggregation of the ganglions was carried out, and neurons were transferred with pipettes into clock glass (15-20 min). The isolated neurons were incubated for 12-18 hours at room temperature (18-24 °C) in a plastic chamber (volume 5 ml).

Microelectrodes for recording the membrane potential were pulled from filaments of borosilicate glass (1.5 mm Ø) and had a resistance of 7-15 MΩ when filled with 2.5 M KCl. All the data were collected by analog-to-digital converter (L-CARD, Russia). All experiments were performed at room temperature (18-24 °C).

Peak values of depolarization or hyperpolarization of the neuronal membrane produced by application of physiological concentrations of caffeine or CsA were measured and the peak amplitude of MP changes was expressed as the percentage of the pretreatment value. The time from application of physiological concentrations of caffeine or CsA to appearance of the first peak of MP change was also measured.

Data treatment was done with the Excel 7.0. Results were presented as mean±SEM. Differences between groups were estimated using the Mann-Whitney U test.

Cyclosporin A in the concentration of 20mM and caffeine in the concentration of 5mM (physiological concentrations) were used. Cyclosporin A (Sundimmun) was obtained from Novartis (Switzerland) and caffeine from ICN (USA).

Low concentration solutions of caffeine and cyclosporin A were produced using the routine method for homeopathic drugs by "Materia medica" Company. C6 dilution (corresponding to the dilution of $1:10^{12}$) was used in the experiments.

4. RESULTS

4.1. Effects of pretreatment with low caffeine concentrations on neuronal reaction produced by application of physiological caffeine concentrations

For considering the possibility that neuronal responses to exogenous ligands might be regulated by low concentrations of the same substances, the soma of the neuron was exposed to solutions of the substances twice. At first, neurons were treated with a saline solution containing low concentrations and then physiological ligand concentrations. The neurons were exposed to solutions of low ligand concentrations under different conditions and after different periods of cultivation: under the stressful actions of mechanical disaggregation of the ganglions and isolation of neurons or after 12-18 hours of cultivation.

Caffeine modulates the signal (cAMP- and Ca^{2+}-dependent) and energy systems of cells and causes an increase of neuronal activity (6,13). We assumed that interactions of caffeine with several types of receptors increased the opportunity to detect the effect of low concentrations of caffeine in electrophysiological experiments.

At first, we were interested to see how physiological caffeine concentrations acted upon the membrane potential of isolated neurons. For this purpose, a recording microelectrode was inserted into the neuron, and 50 μl of caffeine was applied on the neuron by the closely located micropipette (see Figure 1). Examples of neuron responses to the application of caffeine are given in Figure 2a. Physiological caffeine concentration (5 mM) caused reversible MP changes in all the neurons studied. Depolarization and hyperpolarization of the neuronal membrane was observed. MP changes of were observed during first 5-10 min. Then the amplitude of changes decreased and MP returned to the initial value. The first peak of MP change was observed on the average in 99.1±14.4 sec. Neurons with peak amplitude of MP changes of more than 30% predominated in the control group (control group N 1). A diagram of distribution of the number of cells according to the peak amplitude of MP changes caused by application of caffeine is presented in Figure 3.

Low caffeine concentration did not cause any MP changes of isolated neuron soma. Treatment of neurons with caffeine solutions at different periods of cultivation allowed detecting the effects of low caffeine concentrations. It was found that low ligand concentrations had effects on neuronal responses if the mechanical disaggregation of the ganglions and isolation of neurons

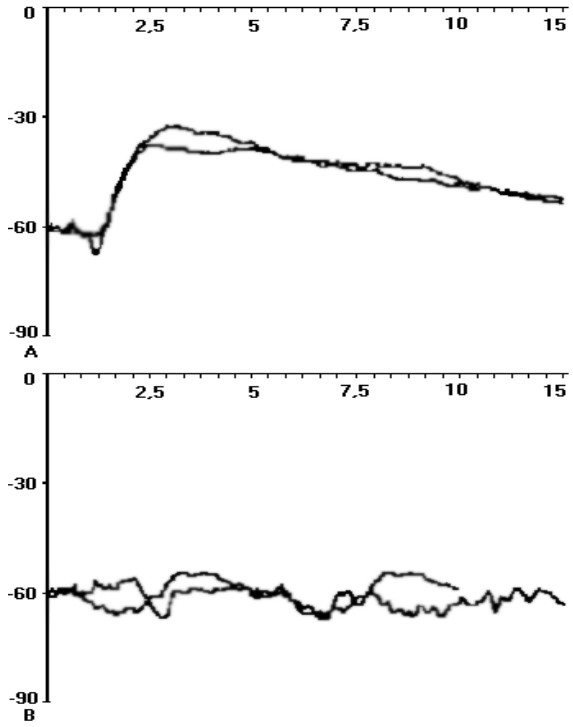

Figure 2. Examples of membrane potential changes caused by application of solutions of physiological caffeine concentrations. The application of solutions of physiological caffeine concentrations and the recording of membrane potential of isolated neurons were conducted in all groups after 12—18 hours of cultivation. The neurons of control group were not pretreated by low caffeine concentration (2a). The neurons of the second group were pretreated by low caffeine concentration 15—20 min during the mechanical disaggregation of the ganglions and isolation of neurons (in the stress condition) (2b). Axes: Y - value of membrane potential in mV. X - time from beginning of application of caffeine, minutes.

was performed in a saline solution containing low caffeine concentration. It should be emphasized here that the mechanical disaggregation of the ganglions was carried out during 15-20 min. The isolated neurons were carefully washed in clock glasses with saline solution and transferred with a pipette into Petri dishes for cultivation. Further experiment was carried out after 12-18 hours of cultivation as in control group. Examples of neuron responses to the application of caffeine are given in Figure 2b. Pretreatment of the neurons with low caffeine concentration decreased the amplitude of MP changes caused by application of physiological caffeine concentrations. In the control group N1 of neurons the peak amplitude of MP changes varied from 7 % to 103 % (on the average 58.4±5.1%; 30 neurons). In the group of neurons pretreated with low caffeine concentrations during disaggregation of the ganglions, the peak amplitude of MP changes varied from 6 % to 100 % (on the average 27.6±3.8%; 32 neurons). The average peak amplitude of MP changes was decreased by 30.8% in comparison with the control group N1. The majority of neurons with small amplitude of MP changes (6-30%) were detected in this group. The percentage of these neurons was 78% of the total number of the cells in the group. The first peak of MP change was observed on the average in 96.5±11.3 sec. A typical course of MP changes of these groups is represented in Figure 2a, 2b. The average baseline values of MP of these groups did not differ (63.9±5.4 in the control; 64.3±4.3 in the experimental group). The differences of peak amplitude of MP changes between groups were statistically significant (P_U<0.001). A diagram of distribution of the number of cells according to the peak amplitude of MP changes caused by application of caffeine is presented in Figure 3.

Pretreatment of neurons during 40 min with a saline solution containing low caffeine concentrations after 12-18 hours of cultivation did not influence essentially the neuronal reactions caused by application of physiological caffeine concentrations. The average peak amplitude of MP changes was 57.2±4.7% (21 neurons) in the group of cells incubated during 40 min immediately before MP registration in a saline solution containing low caffeine concentrations (58.4±5.1% in control group N1). A diagram of distribution of the number of cells according to the peak amplitude of MP changes caused by application of caffeine is presented in Figure 3.

4.2. Effects of pretreatment with low cyclosporin A concentrations on neuronal reaction produced by application of physiological cyclosporin A concentrations

Caffeine produces excitatory effects on targets and causes an increase of neuronal activity. For considering the possibility that responses of neurons might be regulated by low concentrations of substances having an inhibitory effect, the cells were exposed to cyclosporin A (CsA). The experiment was performed like that which revealed the action of low caffeine concentrations, but instead of alkaloid, CsA peptide was used. This peptide interacts with Ca^{2+}-release channel of sarcoplasmic reticulum, one of types of mitochondria pores and cell phosphatases (7,17). CsA has an inhibitory effect on these targets.

At first, we were interested to see how physiological CsA concentrations acted upon the MP of isolated neurons. CsA induced reversible MP changes in all the neurons studied. Examples of neuron responses to application of CsA are given in Figure 4a. In this group (control group N2) of neurons the peak amplitude of MP changes varied from 32% to 108% (on the average 68.4±12.3%; 11 neurons). The first peak of MP change was observed on the average in 162±24.3 sec.

Mechanical disaggregation of the ganglions and isolation of the neurons of this group of cells was performed in a saline solution containing a low CsA concentration. Examples of neuron responses to application of CsA are given in Figure 4b. Pretreatment of neurons with low CsA concentration decreased the amplitude of MP changes caused by application of physiological CsA concentrations. In the group of neurons pretreated with low

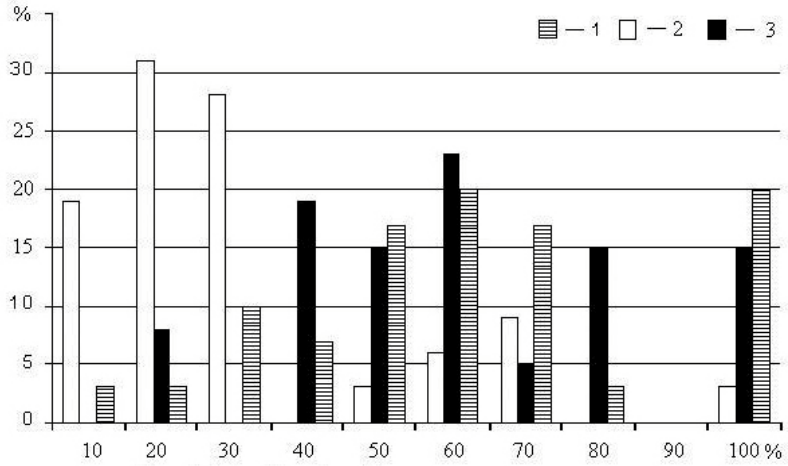

Figure 3. Distribution of the number of neurons according to the peak amplitude of membrane potential changes caused by application of caffeine. The application of solutions of physiological caffeine concentrations and the recording of membrane potential of isolated neurons were conducted in all groups after 12—18 hours of cultivation. The neurons of first group were not pretreated by low caffeine concentration. The neurons of the second group were pretreated in a saline solution containing low caffeine concentration 15—20 min during the mechanical disaggregation of the ganglions and isolation of neurons (in the stress condition). The neurons of the third group were incubated during 40 min immediately before MP registration in a saline solution containing low caffeine concentrations. Axes: Y - the number of neurons (% of the total number of cells in the group). X - peak amplitude of membrane potential changes, percent of the pretreatment value

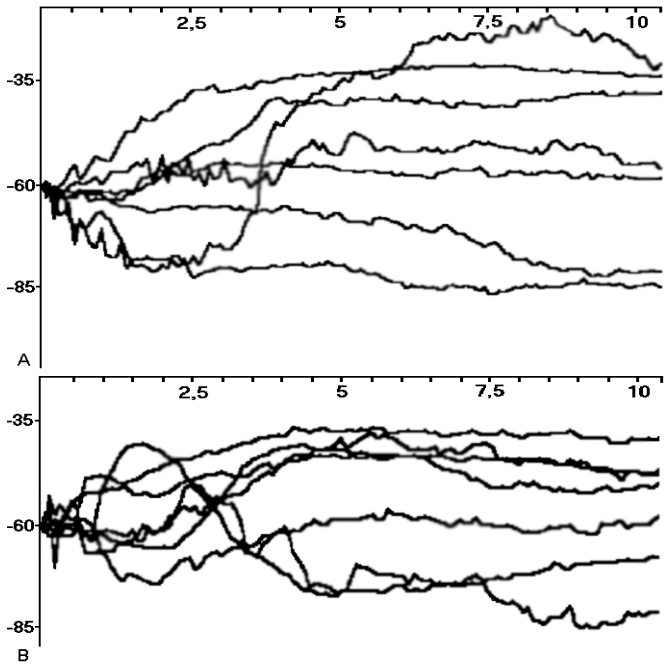

Figure 4. Examples of membrane potential changes caused by application of solutions of physiological cyclosporin A concentrations. The application of solutions of physiological cyclosporin A concentrations and the recording of membrane potential of isolated neurons were conducted in all groups after 12—18 hours of cultivation. The neurons of control group were not pretreated with low cyclosporin A concentration (4a). The neurons of the second group were pretreated in a saline solution containing low cyclosporin A concentration 15—20 min during the mechanical disaggregation of the ganglions and isolation of neurons (in the stress condition) (4b). Axes: Y - value of membrane potential in mV. X - time from beginning of application cyclosporin A, minutes.

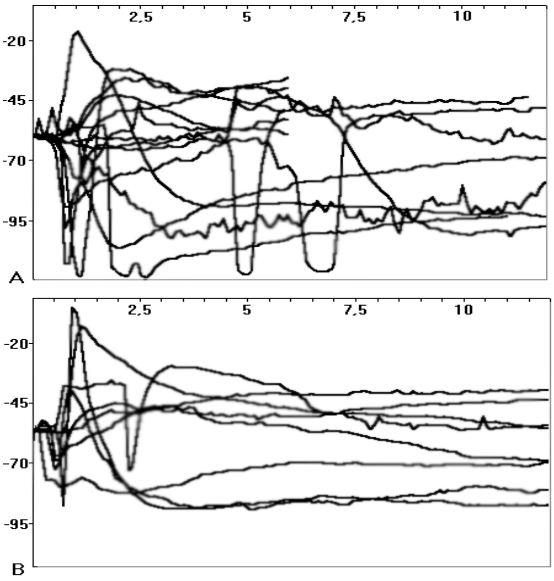

Figure 5. Examples of membrane potential changes caused by application of solutions of physiological caffeine concentrations. The application of solutions of physiological caffeine concentrations and the recording of membrane potential of isolated neurons were conducted in all groups after 12—18 hours of cultivation. The neurons of control group were incubated during 30 min in saline solution containing physiological cyclosporin A concentration (5a). The neurons of the second group were pretreated in a saline solution containing low cyclosporin A concentration 15—20 min during the mechanical disaggregation of the ganglions and isolation of neurons (in the stress condition) (5b). Axes: Y - value of membrane potential in mV. X - time from beginning of application caffeine, minutes.

CsA concentrations during disaggregation of cells, the peak amplitude of MP changes varied from 15% to 88% (on the average 39.3±9.4%; 7 neurons). In this group of neurons, the average peak amplitude of MP changes was decreased by 29.1% in comparison with the control group. The first peak of MP change was observed on the average in 90±27.4 sec in this group of neurons. The differences of peak amplitude of MP changes between these groups were statistically significant (P_U<0.001).

Pretreatment of neurons free from stressful influences with a saline solution containing low CsA concentrations at the other periods of cultivation did not affect essentially the neuronal reactions to application of physiological CsA concentrations.

4.3. Effects of pretreatment with low cyclosporin A concentrations on neuronal reaction produced by application of physiological caffeine concentrations

For considering the possibility that the responses of neurons to physiological concentrations of one type of ligands might be regulated by low concentrations of other ligands, the neuron soma was successively exposed to different ligands having a common target (ryanodine receptors) (7,13,17). The experiments were performed like that in which the action of low concentrations of caffeine or CsA was revealed. However, the neurons were first exposed to low CsA concentrations, and then to physiological caffeine concentrations.

We examined also the response of the neurons incubated in CsA solution of physiological concentration (30 min) to application of physiological caffeine concentration. Examples of neuronal responses of this group are shown in Figure 5a. In this group (control group N3) of neurons, the average peak amplitude of MP change was 78±12.5% (13 neurons). The appearance of first peak of MP change was observed on the average in 82.5±22.6 sec.

The next group of neurons was pretreated with a saline solution containing low CsA concentrations during mechanical disaggregation of the ganglions and isolation of neurons. After this pretreatment of neurons with low CsA concentration, the average peak amplitude of MP change to application of physiological caffeine concentration was 51.9±9.5 % (8 neurons). The examples of neuronal responses of this group are given in Figure 5b. The first peak of MP change was observed on the average in 35.5±4.8 sec in this group. The control group N1 of cells (without pretreatment) and the group of the neurons incubated in solution of physiological CsA concentration demonstrated the first peak of MP change on the average in 99.1±14.4 sec and 82.5±22.6 sec, respectively.

5. DISCUSSION

An important conclusion of this study is that the stressed cells are more sensitive to the action of low ligand concentrations. In our experiments, the effects of the pretreatment of cells with low ligand concentrations appeared as a decrease of the neuronal response to physiological concentrations of each of two ligands studied (caffeine, CsA). Cases of development of tolerance *in vivo* after prior moderate actions are known. The development of ischemic tolerance after a prior mild ischemia *in vivo* suggests the existence of some endogenous mechanisms mediating long-term protection against ischemia (9,16). *In vitro* experiments using organotypic slice cultures of rat hippocampus revealed that the extracellular serine protease thrombin and its receptors are endogenous mediators of neuronal protection against brain ischemia (3,20). Very low concentrations of thrombin (50 pM) protected hippocampal neurons and astrocytes from a variety of cellular injuries, including hypoglycemia, growth supplement deprivation, oxidative stress, and beta-amyloid toxicity (15,21), while high concentrations of thrombin induced apoptosis in the same cells (4,1518,21). The results presented here as well as the data of other authors (3,5,10,12,20) show the physiological (information) value of low concentrations of exogenous and endogenous substances. Unfortunately, pharmacological and biochemical studies of changes induced by the interaction of low ligand concentrations with the known cell receptors do not allow determining the degree in which the second messengers are involved in the

pathways mediating the long-term plastic responses of cell. The responses of the cells depend not only on the concentration of substances, but also the amplitude, the duration and the succession of activation of second messengers, the interactions of signal transduction pathways. The difference in activation conditions of the second messengers may also explain the different responses caused by equal concentrations of ligands (22). The difference in the results obtained with pretreatment of the neurons with low concentrations of caffeine or CsA at different periods of cultivation may be interpreted as follows. Neurons experiencing stress may express a different background of downstream effector proteins so that connections (perhaps, structural ones) of these effector proteins with caffeine or CsA receptors arise. It has been shown that enzymatic treatment of neurons disturbs the ionic homeostasis, the acid-alkaline balance and activates regeneration processes (11). We found that in these conditions the neurons were more sensitive to the action of low ligand concentrations. The average peak amplitude of MP changes was 57.2±4.7% in the group of cells incubated for 40 min immediately before MP registration in a saline solution containing low caffeine concentration and it was 58.4±5.1% in the control group (without pretreatment). In the group of neurons experiencing stress (during disaggregation of cells) and the influence of low caffeine concentration, the amplitude of MP changes decreased on the average by 30.8% in comparison with the control group. In this group, more neurons with small (6-30%) peak amplitude of MP changes were detected (Figure 2b). The percentage of these neurons was 78% of the total number of cells in the group.

Caffeine is an alkaloid which modulates signal (cAMP- and Ca^{2+}-dependent) and energy systems of cells and increases the neuronal activity (1,6,8,13,14). Protein CsA has a blocking effect on the shared target with caffeine (ryanodine receptors of sarcoplasmic reticulum) (7,17). However, pretreatment of stressed neurons with low CsA concentrations changed the neuronal responses to application of physiological concentrations of the protein solution in a similar way. The average peak amplitude of MP changes decreased by 29.1% in this group compared with the control group. Further experiments were designed to reveal the possibility that the neuronal responses to physiological concentrations of one ligand might be regulated by low concentrations of other ligands having the same target (ryanodine receptors). For this purpose, neurons were treated with solutions containing low CsA concentrations and then those containing physiological caffeine concentrations. It was found that in the group of neurons treated with low CsA concentrations and then with physiological caffeine concentrations the average peak amplitude of MP changes was 51.9±9.5%, and in control groups of cells (without pretreatment) it was 58.4±5.1%. However, in this group the first peak of MP change was observed on the average in 35.5±4.8 sec, in 2.8 times faster than in control groups of cells (without pretreatment).

The changes of MP caused by caffeine application in the majority of neurons pretreated with low caffeine concentration represented repetitive jumps MP of small amplitude. The neurons of the control group showed a significant change of MP to caffeine application. A typical course of MP changes for these groups is presented in Figure 2a, 2b. It is important to note that thrombin initiates generation of inositol 1,4,5-trisphosphate, which regulates Ca^{2+} intracellular concentration (2). A low thrombin concentration induced single or repetitive Ca^{2+}- spikes in hippocampal CA1 neurons that were loaded with fura-2. A high thrombin concentration caused a delayed single Ca^{2+}-spike followed by a sustained plateau phase (20).

The findings presented here permit us to assume a significant role of preliminary action of low ligand concentrations in the development of changes of the response to application of high concentrations. Basing on the experimental data, we propose the following model of plastic responses. Low ligand concentrations do not cause such responses as their physiological concentrations, because the physiological ligand concentrations provide for a prolonged interaction of ligands with the majority of the cell receptors. Low ligand concentrations may provide only discontinuous interaction of ligands with cell receptors. Repetitive rare interactions of ligands with receptors at the cellular level develop conditions similar those induced by repetitive stimuli in classical learning processes. These repetitive reactions of receptors with ligands may lead to modifications of the amplitude, duration or speed of activation of the secondary mediator systems or microreorganizations of interactions of the receptor and effector systems of the cell. Application of high concentrations (of the same ligands or ligands that have similar targets) after the action of low ligand concentrations permit disclosing plastic changes of neuronal responses.

6. ACKNOWLEDGEMENTS

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Send correspondence to: Dr. A.S. Ratushnyak, Technological Institute of Digital Techniques SB RAS, Institutckai street, 6, 630090, Novosibirsk, Russia. Tel. 7(3832)343-918, E-mail: Ratush@kti.nsc.ru