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MEMBRANE AND SYNAPTIC EFFECTS OF ANTI-S-100 ARE PREVENTED BY THE SAME ANTIBODIES IN LOW CONCENTRATIONS

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1. ABSTRACT

Effects of antiserum to S-100 protein (AS-100) in high (HC, corresponding to antiserum dilution 1:5 – 1:50) and low (LC, $1:10^{12}$) concentrations were studied in identified snail neurons and rat hippocampal slices. HC-AS-100 changed the frequency of action potential generation in spontaneously active neurons and blocked formation of long-term potentiation in mossy fiber synapses. LC-AS-100 alone did not affect any characteristic measured in our experiments, but 20 min pre-incubation of snail ganglia or hippocampal slices with LC-AS-100 abolished the effects of the same antibodies in HC. Simultaneous addition of both LC and HC did not prevent the development of HC-AS-100 effects. It seems possible that LC-AS-100 is capable of interaction with neuronal cells modifying their reaction to HC-AS-100.

2. INTRODUCTION

Antibodies to proteins of the nervous tissue including calcium binding regulatory S-100 protein are widely used in the studies of functional role of these proteins at different levels of neuronal organization – from individual neurons to whole brain (1,2). On the other hand, autoantibodies to brain proteins have been identified in the majority of neurological and psychic disorders, and the data obtained in the studies of the antibodies effects could be of great value for the analysis of their involvement in pathological processes at the cellular level.

Effective concentrations of antibodies were rather high in the experiment mentioned above. Thus the question arose if antibodies in significantly lower quantities were capable of modifying nerve cell activity. In our experiments we used low concentrations of antibodies to S-100 and well-studied models such as identified snail neurons and long-term potentiation in rat hippocampal slices.

3. MATERIALS AND METHODS

3.1. Experimental and control serum

Monospecific antiserum to S-100 protein (A-S-100) and non-immune rabbit serum were a generous gift of Dr. S.M.Sviridov. Procedures of S-100 purification and antiserum preparation were described earlier (3). Low concentrations of antibodies were produced using a routine method for homeopathic drugs by «Materia medica» company staff. 6C dilution (corresponding to dilution $1:10^{12}$) was used in the experiments. Nonimmune rabbit serum served as control in the experiments with "high" concentrations of A-S-100.

3.2. Experiments on identified neurons from subesophageal complex of ganglia of terrestrial snails

For our experiments we used snails *Helix lucorum* of Crimea population uniform with respect to weight and size. The snails were kept in active state in a glass terrarium with a humid atmosphere at room temperature and excess of food for two weeks or more before the experiments. Experiments were performed on identified spontaneously active neurons in isolated preparations of subesophageal ganglia (4). Prior to the

preparation procedure snails were anesthetized by immersing in the ice-cooled water for 20-30 min and then the ganglia complex was isolated (5). All measurements were performed at room temperature (20-22^{o}C) using intracellular 5-40 MO glass microelectrodes filled with 2.5 M KCl. The tip potential was checked before and after the experiment. The electrical activity of neurons was observed on an oscilloscope and recorded on-line using a 12- bit analog-digital converter.

During microelectrode study the following parameters of nervous cells were recorded: frequency of action potential generation (N, number of pulses per minute), membrane resting potential (Vm) and threshold of action potential generation (Vt). Membrane resting potential and action potential generation threshold were measured in millivolts (mV). Changes in the frequency of action potential generation were calculated in percent as 100x Ne/Ni, where Ne was the frequency in experimental solution and Ni – initial frequency in saline prior to application of experimental solution. Statistical analysis of the results was made using Student's t-test and Mann-Whitney test. The average values of measured characteristics with standard errors M±SEM were calculated. Besides, these criteria for conjugated pairs were used to verify the reliability of the mean value difference in neuron parameters between different groups of animals.

The following solutions were used in experiments:

- saline solution for snail (SS) (in mM): NaCl - 80, KCl - 4, $CaCl_2$ - 7, $MgCl_2$ - 5, $NaHCO_3$ - 5 (or Tris - 5), pH - 7.6 -7.8;
- antibodies to S-100 protein (A-S-100) – antiserum to S-100 protein, diluted in proportion 1:5 by SS, which corresponded to total protein content of 12 mg/ml ("high" concentration of A-S-100);
- antibodies to S-100 protein in low concentration (LC-A-S-100) – 5 ml of SS + 1 drop (20µl) of A-S-100C6 dilution ;
- in the study of the joint effects of AS-100 in "high" and low concentrations 20 µl of A-S-100C6 were added to A-S-100 in "high" concentration (LC-A-S-100 + HC-A-S-100).

The schedules of the experiments were as follows:

- when the effects of A-S-100 in "high" or low concentrations were studied we first recorded the membrane characteristics of identified neurons in saline solution (SS), then substituted SS by HC-A-S-100 or LC-A-S-100 and continued the registration for another 30 min;
- when the joint effects of AS-100 in "high" and low concentrations were studied, SS was substituted by LC-A-S-100 + HC-A-S-100 or first by LC-A-S-100 only and then by LC-A-S-100 + HC-A-S-100. In this case the registration was continued for 20 min.
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The series of experiments were carried out by the following scheme: 1) 20 min in SS $\rightarrow$ 30 min in HC-A-S-100 or LC-A-S-100; 2) 20 min in SS $\rightarrow$ 20 min in LC-A-S-100 $\rightarrow$ 20 min in LC-A-S-100 + HC-A-S-100.

3.3. Long-term potentiation (LTP) in rat hippocampal slices

Transverse slices, 400 microns thick, were cut from the hippocampus of adult Wistar rats and placed in a chamber with artificial cerebrospinal fluid (ACSF) which contained (in mM): NaCl, 124; KCl, 4.9; KH_2PO_4, 1.2; $MgSO_4$, 1.3; $CaCl_2$, 2.5; $NaHCO_3$, 25.6; D-glucose, 10; (pH 7.5). ACSF was saturated with 95% O_2 / 5% CO_2, perfused at 1-2 ml/min and kept at 36^{o} C. Slices were kept in a superfusion chamber at least for 40 min after preparation to allow their energetic and functional recovery.

LTP was examined in mossy fibers/CA3 pathway. Extracellular recordings of field excitatory postsynaptic potentials (fEPSPs) were performed by low resistance glass microelectrode filled with 2.5M NaCl and inserted into apical dendritic region of CA3 pyramidal cells. fEPSPs were evoked by stimulating mossy fibers with elecrolytically sharpened and insulated bipolar tungsten electrode with rectangular voltage pulses of 0.2 msec. Test stimuli of 10-30V amplitude were applied at intervals of 5 min.

LTP was induced by 100 Hz-stimulus trains each containing 3 bursts of 100 pulses with 2-sec interval between pulses and 10 min interval between the trains. LTP was measured as the ratio of fEPSP amplitude recorded 60 min after tetanic stimulation to that of the control. Responses were filtered at 1 Hz - 10 kHz, digitized on-line (DigiData 1200, Axon Instruments Inc.), stored, and subsequently analyzed using Pclamp 6 program (Axon Instruments Inc.).

To analyze the effects of HC-A-S-100 or LC-A-S-100 we used the following schedule of the experiment: 1or 2 slices of each series were tetanized and further experiments on the slices of this series were performed only if LTP formation was observed.

200 microlitre of AS-100 or non-immune rabbit serum were added to ACSF in experimental chamber (final dilution - 1:50) and after 20 min of incubation evoked potentials were recorded followed by tetanization. fEPSPs were registered for 60 min after tetanization.

In the study of the effects of LC-A-S-100, 40 microlitre of AS-100C6 were added to experimental chamber and the same schedule of the experiment was used.

In the study of the effects of joint administration, both preparations (200 microlitre of A-S-100 and 40 microlitre of A-S-100C6) were added simultaneously or 10-20 min pre-incubation with LC-A-S-100 was used prior to A-S-100 application.

As in some experiments 40% ethanol was used for stabilization of A-S-100C6 solution, additional experiments were carried out using low concentrations of ethanol as the reference solution.

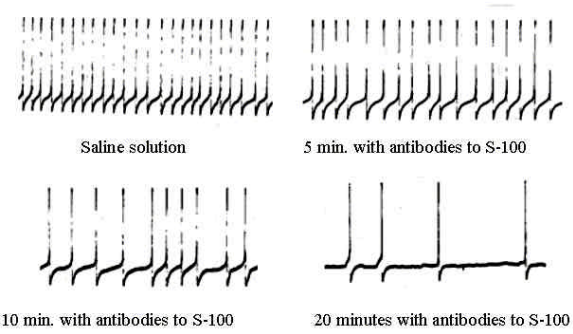

Figure 1. Effect of AS-100 in 1:5 dilution on frequency of action potential generation in spontaneously active cells V1, V3, V17 and RPa6 in ganglion of snail.

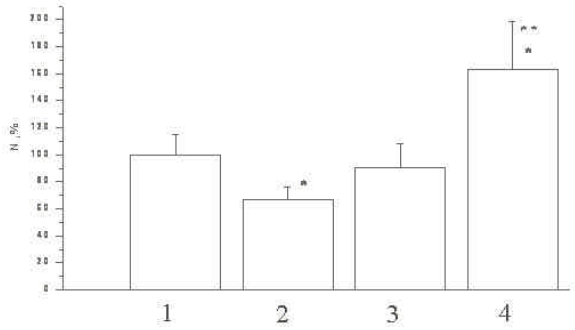

Figure 2. Effects of antibodies to S-100 protein in high (HC-A-S-100) and low (LC-A-S-100) concentrations, and their joint administration (HC-A-S-100+LC-A-S-100) on a frequency of action potential generation in neurons V1,V17. 1 – physiological saline for invertebrates, 2 – HC-A-S-100, 3 - LC-A-S-100, 4 - HC-A-S-100 + LC-A-S-100. * - reliable difference from SS; ** - reliable difference from HC-A-S-100.

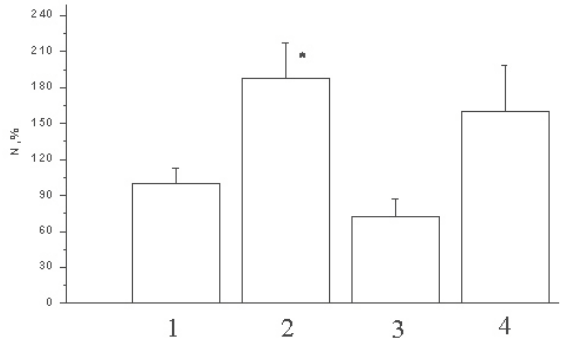

Figure 3. Effects of antibodies to S-100 protein in high (HC-AS-100) and low (LC-AS-100) concentrations, and their joint administration (HC-AS-100+LC-AS-100) on a frequency of action potential generation in neurons V4,V6. 1 – physiological saline for invertebrates, 2 – HC-A-S-100, 3 - LC-A-S-100, 4 - HC-A-S-100 + LC-A-S-100. * - reliable difference from SS; ** - reliable difference from HC-AS-100.

4. RESULTS

4.1. The effects of antibodies to S-100 in "high" and low concentrations on electrical characteristics of snail identified neurons

In preliminary series of experiments two types of neurons in visceral and right parietal ganglia were identified responding to A-S-100 ("high" concentrations) application in different manner.

In spontaneously active cells V1, V3, V17 and RPa6 addition of A-S-100 led to a decrease in frequency of action potential generation (Figure 1, 2). On the contrary, in cells V4, V6 and some other an increase in the frequency of action potential generation was observed (Figure 3). Substitution of SS by LC-A-S-100 did not cause any significant changes in the frequency of action potential generation in both types of cells (Figure 2, 3).

Pre-incubation of neurons with LC-AS-100 for 20 min abolished the effects of A-S-100 in "high" concentration (substitution of LC-A-S-100 by LC-A-S-100 + HC-A-S-100), while simultaneous addition of both concentrations resulted in the development of A-S-100 effects (Figure 2). In some cases pre-incubation with LD-A-S-100 caused opposite effects of "high" concentrations of antibodies: a significant increase in the frequency of action potential generation was observed in the first and a decrease - in the second group of neurons (Figure 3). We did not find any changes in the value of membrane resting potential in both groups of cells when HC-A-S-100 and LC-A-S-100 were added simultaneously. Application of A-S-100 after 20 min pre-incubation in LC-A-S-100 led to a significant heightening of the threshold of action potential generation in neurons V1 and V17 (Figure 4).

4.2. Effects of antibodies to S-100 protein in "high" and low concentrations on LTP formation in rat hippocampal slices

Non-immune rabbit serum, antiserum to S-100 protein (A-S-100) or LC-A-S-100 as well as the reference solution produced no effect on baseline charateristics of field excitatory postsynaptic potentials (fEPSPs) amplitude. Standard records of fEPSP prior to and 40 min after tetanization are shown in Figure 5 demonstrating LTP formation in mossy fibers – CA3 pyramides synapses. 20 min pre-incubation with A-S-100 blocked LTP induction while non-immune rabbit serum did not affect the development of LTP in rat hippocampal slices (Figure 6B, C). The effect of A-S-100 on LTP formation in Shaffer collateral – CA1 synapses was studied in detail by Lewis and Teyler (6). As S-100 is a calcium-binding protein widespread in the nervous tissue and LTP is a calcium dependent process, it seems reasonable that S-100 protein is involved in LTP development in various synaptic systems. This assumption is also confirmed by our earlier findings showing the presence of S-100 protein in central synapses (7) and an increase in membrane-bound S-100 content during LTP formation in hippocampal slices (8).

Incubation with LC-AS-100 did not affect LTP formation (Figure 6D).

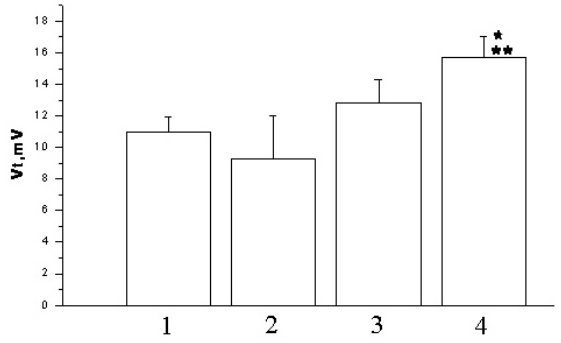

Figure 4. Effects of antibodies to S-100 protein in high (HC-AS-100) and low (LC-AS-100) concentrations, and their joint administration (HC-AS-100+LC-AS-100) on a threshold of action potentials generation of neurons V1,V17. 1 – physiological saline for invertebrates, 2 – HC-A-S-100, 3 - LC-A-S-100, 4 - HC-A-S-100 + LC-A-S-100. * - reliable difference from SS; ** - reliable difference from HC-AS-100.

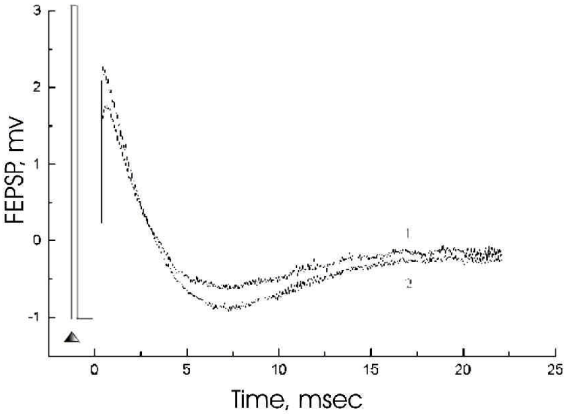

Figure 5. Examples of representative fEPSPs (V) to 20 V test stimulus (Δ) in CA3 hippocampal region: 1 – prior to and 2 – 40 min after tetanization procedure.

When we studied the effect of joint administration of LC-A-S-100 and HC-A-S-100, both solutions were added to experimental chamber simultaneously. In Figure 6E blockage of LTP induction is shown thus indicating that in these conditions LC-A-S-100 does not affect HC-A-S-100 action.

In subsequent modified experiments, low concentrations of antibodies were added prior to A-S-100 administration. After 20 min pre-incubation with LC-A-S-100, A-S-100 was added and the slice was incubated with the A-S-100 in both concentrations for the next 20 min. In this case tetanization of mossy fibers led to LTP formation (Figure 6F).

As in some cases 40% ethanol was used for preparation of A-S-100C6, we analyzed the assumption that the effect could be due to application of ethanol-containing solution that could result in changes of the membrane state or/and disturbance in antibody-antigen interaction. Reference solution did not affect the blockage of LTP induction by A-S-100 when added simultaneously with or prior to A-S-100.

5. DISCUSSION

Our data on the effects of A-S-100 in "high" concentrations coincide completely with those obtained earlier at our laboratory (9) and by other authors (3, 10-12). Ability of A-S-100 to change the excitability of neuronal membrane and to block LTP induction is probably due to the blockage of functional activity of S-100 proteins taking part in regulation of various calcium-depending processes in nervous tissue (13, 14).

In the last years the effects of biologically active substances in low and ultra-low concentration were studied actively. Although the mechanisms of their effects still remain obscure, some characteristic features were established, in particular, the wave character of the association between the active substance dilution and its effect, i.e. "disappearance" and "appearance" of the effect in testing of subsequent dilutions.

Probably, the non-linear character of this "dose-effect" curve can explain the absence of effect of low concentration of A-S-100 alone.

Nevertheless we could show that the influence of low concentration of antibodies results in modification of the "high" concentration effects. Pre-incubation of snail neurons or hippocampal slices with LC-A-S-100 led to the abolition of LTP blockage by HC-A-S-100 as well as to the absence of their effect on generation of action potentials by spontaneously active snail neurons. In both cases this effect that we named "bipathic" was normalizing.

Mechanisms of "bipathic" effect are unclear, but it seems more probable that ultra-low concentrations of antibodies act directly on biological object modifying its further reaction on "high" concentrations of antibodies. The time period necessary for "bipathic" effect realization (pre-incubation with LC-A-S-100) and the absence of this phenomenon in simultaneous application of both concentrations testify for the benefit of this assumption.

The data on the effects of "high" concentrations of antibodies to the endogenous products of nervous tissue including S-100 protein were used in an attempt to explain possible role of autoantibodies in pathogenesis of various neurological and psychic disorders (15). In particular, ability of antibodies to S-100 protein to block LTP formation may contribute to learning and memory impairment in pathology characterized by high levels of autoantibodies to this protein such as alcoholism, vascular dementia and some psychic disorders (16-18).

At the same time it is known that autoantibodies to a wide spectrum of autoantigens are present in the serum of healthy individuals. Such autoantibodies were named "natural" or "naturally occurring". In contrast to autoantibodies identified in pathology, the concentrations of natural autoantibodies are extremely low.

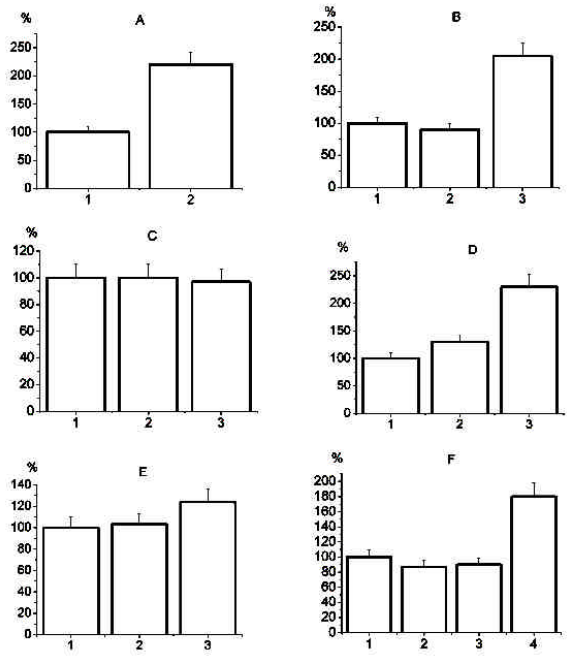

Figure 6. «Bipathic» effect of joint administration of antibodies to S-100 protein in high (HC) and low (LC) concentrations on LTP formation in hippocampal slices. Data are presented as a ratio (in per cent) to fEPSP amplitude prior to tetanization (100%). A. Incubation of slices in standard ACSF. 1 - prior to tetanization, 2 - 40 min after tetanization. B. Pre-incubation with non-immune rabbit serum. 1- ACSF, 2- after 20 min pre-incubation, 3 - 40 min after tetanization. C. Blockage of LTP induction with HC-A-S-100. 1- ACSF, 2 -after 20 min pre-incubation with HC-A-S-100, 3 - 40 min after tetanization. D. Pre-incubation with LC-A-S-100. 1 - ACSF, 2 - after 20 min pre-incubationwith LC-A-S-100, 3 - 40 min after tetanization. E. Joint administration of LC- and HC-A-S-100. Substances were added simultaneously. 1 - ACSF, 2 - 20 min pre-incubation with both concentrations of A-S-100, 3 - 40 min after tetanization. F. Joint administration of LC- and HC-A-S-100. 1 - ACSF, 2 - 20 min pre-incubation with LC-A-S-100, 3 - 20 min incubation with both concentrations prior to tetanization, 4 - 40 min after tetanization.

Usually it is assumed that natural autoantibodies play an important role in maintenance of immune and chemical homeostasis (19, 20), but some authors suppose a wider spectrum of their functions as of regulatory and communicative molecules (21). In some reports the protective and "repairing" effects of autoantibodies and natural autoantibodies in pathological processes are discussed (22, 23). It seems possible that "bipathic" effect concerns the same class of phenomena. Anyway further study of the mechanisms of "bipathic" effect presents doubtless interest for the research of regulatory abilities of antibodies and for the creation of new remedies on the basis of low concentrations of active substances.

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