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THE EFFECT OF SODIUM NITROPRUSSIDE ON RESTING MEMBRANE POTENTIAL OF THE LEECH RETZIUS NERVE CELLS

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We have investigated the effect of sodium nitroprusside (SNP) on the membrane resting potential of the leech (Haemopis sanguisuga) Retzius nerve cells (RNC). The membrane potential of RNC of isolated ganglia was recorded in Ringer solution, in SNP solution during the next 30 minutes and after washing out with Ringer solution. We used 1 mmol/L, 2 mmol/L and 5 mmol/L solutions of SNP. Kruskal-Wallis ANOVA test was used to compare the fall of membrane potential of the leech Retzius nerve cells when different SNP concentrations were used. There was no change in the membrane resting potential of the leech Retzius nerve cells with 1 mmol/L concentration of SNP whilst 2 mmol/L concentration of SNP induced hyperpolarization during the first 20 minutes. The highest concentration of 5 mmol/L SNP induced hyperpolarization in 50% of cells during the first minute and during the next 10 minutes in the other 50%. The significant fall of membrane potential was recorded with 5mmol/L SNP concentration ($p<0.05$).

The SNP induced hyperpolarization of RNC might be the effect of this NO donor on the potassium channels of leech RNC.

Key words: nitric oxide, resting membrane potential, Retzius nerve cells, sodium nitroprusside

INTRODUCTION

Nitric oxide (NO) is a simple radical gas, which forms covalent bonds fairly easily. It is not stored in the cells; it diffuses freely from its site of formation and does not depend for its action on a specific receptor on the target cells. NO is soluble both in water and lipid, and its half-life *in situ* is a few seconds (Shinoda and Whittle, 2001).

In the mammalian organism, NO is synthesized in several types of cells, such as neurons (Centonze *et al*., 2003), microglia, endothelial cells, macrophages and natural killer cells from L-arginine by a family of three isoenzymes termed nitric oxide synthases (NOSs): endothelial (eNOS), neural (nNOS) and inducible NO synthase (iNOS). Endothelial and neural NO synthases are constitutive isoforms (cNOS) which appear to be the "physiological" forms of

the enzyme. Its activity is increased postsynaptically by stimuli such as acetylcholine – Ach (endothelial cells) and glutamate (neuronal cells). Receptor activation by these stimuli increases calcium influx, which in turn activates calcium-dependent NOS in these generator cells. cNOS synthesizes small amounts of NO, which acts as a signaling molecule. iNOS is calcium independent, and appears to be the "pathophysiological" form of the enzyme since it produces much greater amounts of NO than cNOS and a high concentration of NO can be toxic to cells (Shinoda and Whittle, 2001).

The gas NO is a messenger that modulates neuronal function. Endogenous NO modulates the release of several neurotransmitters, such as acetylcholine, catecholamines, excitatory and inhibitory amino acids, serotonin, histamine, and adenosine. Enhanced NO level in the tissue increases the release of neurotransmitters in most cases, although decreasing effects have also been observed. NO primarily operates as an intercellular messenger. It diffuses rapidly and influences NO-responsive target cells probably within surprisingly extended spatial limits of approximately 0.3–0.4 mm (Wood and Garthwaite, 1994; Lancaster, 1997). The rapid diffusion of NO and its capacity to modulate transduction pathways and neuronal activity attribute to this gas the function of a universal messenger in the brain. Even in areas where no acute effects of NO on excitability are manifest, NO may exert a significant function in long-term potentiation or long-term depression (Maffei *et al*., 2003; Prast and Philippu, 2001). Soon after the discovery of NO synthesis in brain tissue, it has been suggested that NO acts as a retrograde messenger which influences synaptic transmission in the presynaptic cell and promotes synaptic plasticity. NO modulates stimulation-evoked field excitatory potentials and firing rates recorded from single neurons in almost all brain regions and in the spinal cord. The unique feature of NO to rapidly diffuse and to reach cells in a relatively extended sphere suggests that NO may exert important functions even in brain structures with low density of nitrergic neurons. Besides the hippocampus, NO is involved in activity-dependent synaptic plasticity in several other brain regions, which also possess key roles in cognitive, emotional and/or behavioral functions (Prast and Philippu, 2001). On the basis of recently published data, NO is involved in a completely new form of nonsynaptic communication in the CNS, which is established, with the help of NO, between glutamatergic and monoaminergic neurons, and which does not require specific receptors (Kiss and Vizi, 2001). NO is also involved in neuronal development (Tagliaferro *et al*., 2003) and neuronal differentiation. The signalling pathway triggered by NO in physiological processes involves the activation of soluble guanylate cyclase and S-nitrosylation of proteins, and, as recently proposed, nitration of tyrosine residues in proteins (Cappelletti *et al*., 2003). Recent studies suggest that NO may also mediate the switch from proliferation to differentiation during neurogenesis (Gibbs, 2003).

In the cerebellum, NOS is expressed in parallel fibres and within the internal granule cell layer (IGL). During development there are changes in NOS concentration, distribution and activity within the IGL, suggesting NO may play a role in IGL function. NO is a potentially novel mechanism for tuning GABAergic signalling to granule cells and therefore modulating the output of an important

cerebellar circuit (Wall, 2003). The release of endogenous NO is essential for the induction of synaptic plasticity in the cerebellum (Moncada and Higgs, 1991).

Beside studies on mammals there are studies, which were done on leech nerve cells. Damage of the leech or mammalian CNS increases NO production and causes accumulation of phagocytic microglial cells at the injury site. Results of Chen *et al*. (2000) suggest that NO produced at a lesion may be a stop signal for microglia to accumulate there and that it can act on microglia early in their migration. Thus, NO may assume a larger role in nerve repair and recovery from injury by modulating accumulation of microglia, which appear to be important for axonal regeneration. Indirect measurement of NO with the standard citrulline assay (Kumar *et al*., 2001), demonstrated that NO was generated within 30 min after nerve cord injury. Thus, injury activates eNOS already present in the CNS and precedes the accumulation of microglia at the lesion, consistent with the hypothesis that NO acts to stop the migrating microglia at the lesion site. Recent studies on the leech central nervous system (CNS), in which synapse regeneration is successful, have shown that NO generated immediately after injury by eNOS stops migrating microglia at the lesion. The results indicate a new role for NO, directing the microglial cell migration as well as stopping it (Duan *et al.*, 2003).

Beside its roles in the nervous system, NO has very important roles in the neuroendocrine system. NO is biological mediator of the neuroendocrine axis. Neurons with the highest expression of nNOS in the brain are found at various locations in the hypothalamus and, in particular, in the paraventricular and supraoptic nuclei with axons which project to the median eminence and extend into the neural lobe (McCann *et al*., 1996, Prevot *et al*., 2000). NO is secreted at the median eminence (ME), the common termination field for the antehypophysiotropic neurons, under the stimulation of other signaling substances. At the ME, NO stimulates gonadotropin-releasing hormone (GnRH) and corticotropin-releasing hormone (CRH) release from neuroendocrine terminals (Prevot *et al*., 2000). ME can spontaneously release NO and that NO's rhythm of secretion varies distinctly across the estrous cycle (Knauf *et al*., 2001). Chronic NO deficiency is associated with a decreased GnRH in neurosecretory terminals in the external capillary layer of the median eminence, accompanied by a decrease in LH and FSH release from the pituitaries (Barnes *et al*., 2001). NO, by a paracrine mechanism, inhibits prolactin release. Several mechanisms are involved in the inhibitory effect of NO on prolactin release (Velardez *et al*., 2000; Gonzalez and Aguilar, 1999). Oxytocin neurons strongly express NOS and NO inhibits their activity (Bull *et al*., 2003). According to Stern and Ludwig (2001), NO inhibition of neuronal excitability, in oxytocin and vasopressin neurons, involves pre- and postsynaptic potentiation of GABAergic synaptic activity in the supraoptic neurons. On the other hand, Yang and Hatton (1999) suggest that the resulting action of endogenously released NO would be to increase oxytocin and vasopressin release.

We used the horse leeches (*Haemopis sanguisuga*) for further investigation of NO effects. *Haemopis sanguisuga* is a very simple experimental model, easy for delivery and storage. According to the results of Leake *et al*. (1995) there is NOS in

the leech (*Hirudo medicinalis*) CNS. Their hypothesis that NO is involved in neuronal signalling in the leech CNS is based on: a) the presence of fixative resistant NADPH-diaphorase (NADPH-d) in a specific neuronal population in the segmental ganglion; b) direct measurements with NO-sensitive electrodes giving an estimated NO concentration of 400-600 nM; c) biochemical assay for NOS using L-arginine/L-citrulline conversion in the CNS which gave a comparable value to that of the rat cerebellum and d) specific pharmacological effects of the NO precursor L-arginine and a NOS inhibitor (NG-Nitro-L-arginine) in modulation of chemical connections between NADPH-d-positive and NADPH-d-negative neurons. Leech ganglia also have an efficient defence system against reactive oxygen species (Jovanovic and Beleslin, 1997). That's why these neurons are an ideal experimental model for the study of the possible effects of NO on synaptic interactions.

MATERIAL AND METHODS

Our experiment was performed on Retzius nerve cells (RNC) in the abdominal ganglia of horse leeches (*Haemopis sanguisuga*).

Leeches were anesthetized with 10% ethanol solution and then prepared under stereo microscope (Carl Zeiss Jena, enlargement 63 x) according to the dissection procedure of Beleslin and Mihailović (1967). RNC of isolated ganglia were bathed in leech Ringer solution containing (mmol/L): 115 NaCl, 4 KCl, 2 $CaCl_2$, 1.2 Na_2HPO4, 0.3 NaH_2PO4 (pH 7.2). The chamber bath volume was 2 mL. The membrane potential of cells in Ringer solution was recorded. Ringer solution, after 15-20 minutes, was changed with sodium nitroprusside (SNP) within 30s and the membrane potential was registrated during the next 30 minutes (at 1., 10., 20. and 30. minute). We used different SNP concentrations – 1 mmol/L, 2 mmol/L and 5 mmol/L (NRK Inzenjering Beograd). SNP was dissolved in leech Ringer solution (20 mL) immediately prior to use. After 30 minutes exposure to SNP, RNC of isolated ganglia were washed out with Ringer solution. The membrane potential indicating cells' recovery was recorded at the first and the 15^{th} minute after washing out. All experiments were performed at room temperature (20-24 ºC).

The resting membrane potential was recorded intracellularly, using glass microelectrodes manufactured with borosilicate glass capillaries using a puller (Industrial Science Associated Inc.), filled with 3M KCl and having a resistance of about 10MΩ in leech saline. Only microelectrodes with a tip potential less than 5 mV in the artificial solution were selected for use. Microelectrodes were inserted into the cells by a mechanic micromanipulator (W.R.Prior & Co.Ltd, Bishop's Stortford Herts England). Microelectrodes were connected through a Ringer bridge and Ag-AgCl electrodes via a negative capacitance high input resistant amplifier (Bioelectric Instruments) to a computer.

Membrane potential was registrated in respect to the chloride silver electrode in the Ringer solution: it communicates with the test solution in the bath by way of a glass tube filled with 3M KCl in agar. The voltage was filtered with an analog-to-digital converter and stored in a computer for later analysis.

Kruskal-Wallis ANOVA test was used to compare the fall of membrane potential of the leech Retzius nerve cells among different SNP concentrations. Mann-Whitney U test was used to compare the fall of membrane potential of the leech Retzius nerve cells between the start point and subsequent minutes of registration. Comparisons of membrane potentials at the same minute of registration for different SNP concentrations were done using ANOVA and post hoc Bonferroni test when appropriate. All the calculations were performed with the SPSS statistical package (SPSS Inc., Chicago Illinois, USA). A p-value <0.05 was considered statistically significant.

RESULTS

By analysing membrane potential of the leech RNC in different SNP concentrations, the obtained results are presented on Table 1 and Figure 1. The results are presented as the means of membrane potentials and standard deviations. The value of membrane potential at the zero minute is the resting membrane potential, and after that membrane potentials were registered at the 1^{st}, 10^{th}, 20^{th}, 30^{th} minute after SNP replacement.

Table 1. Membrane potentials of Retzius nerve cells in different SNP concentrations

Time (min)	MP (mV) $\bar{x} \pm SD$		
	SNP 1 mmol/L (N=4)	SNP 2 mmol/L (N=7)	SNP 5 mmol/L (N=6)
0’	-37.25 ± 8.38	-38.57 ± 4.75	-35.17 ± 3.97
1’	-36.25 ± 11.03	-43.86 ± 7.88	-45.33 ± 7.34
10’	-38 ± 11.55	-46.43 ± 8.88	-43.83 ± 6.18
20’	-35.33 ± 11.05	-44.14 ± 6.87	-32 ± 9.27
30’	-36.5 ± 13.02	-34.86 ± 7.24	-28.33 ± 8.48
1’reR	-24 ± 10.12	-34.14 ±12.84	-36.5 ± 3.91
15’reR	-36.66 ± 17.24	-32.83 ± 20.67	-29.33 ± 8.45

SNP – sodium nitroprusside; MP – membrane potential; reR – re-replacement of Ringer solution; $\bar{x} \pm SD$ – means ± standard deviation; N – number of samples

Comparing membrane potentials of the leech RNC in Ringer solution before SNP replacement (zero minute) with the results obtained in the other minutes (1^{st}, 10^{th}, 20^{th} and 30^{th}) after 1 mmol/L SNP replacement, we haven’t found changes in the resting membrane potential. On the other hand, 2 mmol/L SNP induced hyperpolarization of RNC during the whole period of 20 minutes after SNP replacement, but without a significant difference. The significant ($p<0.05$, Table 2) fall of membrane potential was recorded with the highest, 5 mmol/L SNP

concentration in the first and 10th minute of registration. This SNP concentration induced hyperpolarization in 50% of cells during the first minute and in the other 50% during the next 10 minutes.

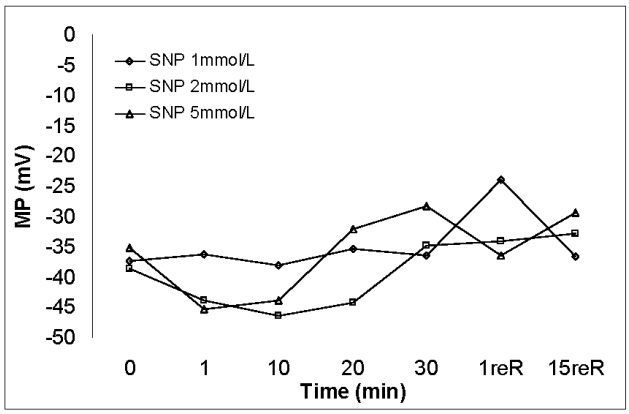

Figure 1. Means of membrane potentials of Retzius nerve cells for different SNP concentrations
Legend: MP – membrane potential of Retzius nerve cells
SNP – sodium nitroprusside
reR – re-replacement of Ringer solution

Table 2. Differences of membrane potential of Retzius nerve cells between zero and 30 minutes of registration in different SNP concentrations

Time (min)	1 mmol/L SNP		2 mmol/L SNP		5 mmol/L SNP	
	Z	p	Z	p	Z	p
0'/1'	0.144	0.889	-1.518	0.154	-2.863	0.0168*
0'/10'	-0.105	0.919	-2.062	0.061	-2.890	0.0160*
0'/20'	0.263	0.802	-1.764	0.103	0.768	0.459
0'/30'	0.096	0.926	1.134	0.278	1.788	0.104
0'/1'reR	1.413	0.252	0.855	0.408	-0.298	0.771
0'/15'reR	0.060	0.954	0.717	0.487	1.529	0.157

SNP – sodium nitroprusside; Z – Mann Whitney U test value; p – statistical difference;
*$p<0.05$; reR – re-replacement of Ringer solution

Comparing membrane potentials of the leech RNC at the same time intervals of registration as for different SNP concentrations, there was no

significant difference (Table 3) after applying the Kruskal-Wallis ANOVA test. The values of membrane potentials were different, but below the level of statistical significance. The value of $p<0.05$ was considered as statistical significant difference.

Table 3. Statistical differences between membrane potentials of Retzius nerve cells for different SNP concentrations at the same time of registration

Time (min)	p		
	SNP 1 mmol/L: SNP 2 mmol/L	SNP 1 mmol/L: SNP 5 mmol/L	SNP 2 mmol/L: SNP 5 mmol/L
0’	1.000	1.000	0.857
1’	0.541	0.373	1.000
10’	0.436	0.953	1.000
20’	0.481	1.000	0.073
30’	1.000	0.571	0.669
1’reR	1.000	1.000	1.000
15’reR	1.000	1.000	1.000

SNP – sodium nitroprusside; p – statistical difference; reR – re-replacement of Ringer solution

DISCUSSION

NO released from many NO-producing cells, which are scarcely distributed in many tissues, may influence neurons in a widely extended area. NO elicits a surprisingly wide range of physiological and pathophysiological effects because NO can act through different mechanisms.

After being released by generator cells, NO stimulates soluble guanylate cyclase (GC) in the target cells, resulting in a rise in cyclic guanosine monophosphate (cGMP) (Shinoda and White, 2001). The selectivity of this messenger is achieved by NO-receptive characteristics of cell components in certain cells, thus being NO target cells (Prast and Philippu, 2001). The activation of soluble GC, S-nitrosylation and nitration of tyrosine residues in proteins are important for the differentiation of neurons (Cappelletti *et al*., 2003), as well as for directing and terminating the microglial cell migration (Duan *et al*., 2003).

In most cases, the toxic or cytotoxic effects of NO are mediated through mechanisms probably independent of cGMP (Murad, 1996), but are caused by iron loss and adverse effects on DNA synthesis, DNA repair and mitochondrial respiration. The concentration and the source of NO are the major factors determining the biological effects of NO. The chemical biology of NO cytotoxicity can be divided into two types of effects, direct and indirect. At low concentrations (<1 μM), the direct effects of NO predominate, while at higher concentrations (>1 μM), the indirect effects prevail. Direct toxic effects are those chemical

reactions in which low levels of NO can react directly to mediate cytostasis. Indirect toxic effects are those chemical reactions mediated by reactive nitrogen oxide species (RNOS) such as peroxy nitrites and nitrogen dioxide, which are formed after interactions with either oxygen or superoxide and which are potentially more toxic than NO itself. The indirect effects can be divided into two types of mechanisms: nitrosative stress and oxidative stress (Shinoda and White, 2001; Davis *et al*., 2001).

According to Änggård (1994), when small amounts of NO are formed as a mediator by the constitutive pathway (nNOS and eNOS) the NO preferentially binds to heme. When large amounts of NO are formed, the molecular targets in the victim cells are Cu-Fe proteins, releasing free Cu^{2+} and Fe^{2+}, and generating O_2 and highly toxic hydroxyl radicals. The net effect is a massive oxidative injury. On the other hand, according to Chiueh (1999), NO is a potent antioxidative agent, because it terminates oxidant stress in the brain by a) suppressing iron-induced generation of hydroxyl radicals (OH) via the Fenton reaction, b) interrupting the chain reaction of lipid peroxidation, c) augmenting the antioxidative potency of reduced glutathione (GSH) and d) inhibiting cysteine proteases.

Beside the mechanisms described previously, NO acts through modulation of ion channel function, too.

The results of Hammarstrom and Gage (1999), suggest that NO may directly increase the activity of neuronal persistent Na^+ channels, but not transient Na^+ channels, through an oxidizing action directly on the channel protein or on a closely associated regulatory protein in the plasma membrane. Direct effects of NO on membrane proteins involving S-nitrosylation and disulfide bond formation are also important. A regulatory mechanism of ion channel activity involving sulfhydryl to disulfide conversion has been described previously and "regulatory thiols" have been demonstrated in K^+ channels (Ruppersberg *et al.*, 1991).

Endothelium-derived NO and reactive oxygen species (ROS) have been proposed to regulate vascular tone by complex mechanisms, including the modulation of ion channel function. In the endothelial function itself, activation of Ca^{2+}-activated K^+ channels (K_{Ca}) plays a crucial role by inducing hyperpolarization, which promotes membrane potential-driven Ca^{2+} influx and Ca^{2+}-dependent synthesis of vasodilatory factors. Direct stimulation of endothelial big K_{Ca} by NO might represent a novel mechanism of autocrine regulation of endothelial function and points to a positive feedback mechanism by promoting hyperpolarization and NO production itself. The ROS-induced inhibition of big K_{Ca} could be part of the cellular mechanisms by which ROS impair endothelium-dependent vasodilation (Brakemeier *et al*., 2003). Demirel *et al*. (1994) conclude that K_{Ca} are important for increasing the concentration of Ca^{2+} at the endothelial surface of aortic strips during Ach stimulation and consequently for the synthesis and release of NO. The opening of the K_{Ca} increases K^+ efflux, hyperpolarizing the endothelial cells and increasing the electrochemical gradient.

Gao *et al*. (2002) investigated the effect of SNP on resting membrane potential and potassium currents of the bronchial smooth muscle cells in rats. They concluded that SNP increased the activities of K_{Ca} and voltage-gated K^+

channels and led to K^+ efflux and hyperpolarization of the cell membrane, resulting in the decrease of cell excitement.

Multiple K^+ channels may mediate the hyperpolarization response to NO in smooth muscles. At least two of these channels may be activated by dual pathways involving direct activation by NO and cGMP-mediated mechanisms (Koh *et al*., 1995). Bolotina *et al*. (1994) have demonstrated that both exogenous NO and endothelium-derived relaxing factor (EDRF) can directly activate K_{Ca}, which could cause hyperpolarization and relaxation of vascular smooth muscle independently of cGMP. Their opinion agrees with the opinion of Buxton *et al*. (2001) who have found that NO-induced relaxation of labouring and non-labouring human myometrium is not mediated by cyclic GMP. However, Yu *et al*. (2003) suggest that the increase of Ca induced K^+ current caused by SNP may be mediated by cGMP via IP3-sensitive calcium pools, and extracellular Ca^{2+} may not be involved in the process.

The results of the study on Aplysia neurons demonstrate that sodium nitroprusside and hydroxylamine (NO donors) inhibit the dopamine (DA)-induced K^+ current and as well as that SNP inhibits the ACh-induced K^+ current. The mechanism of NO inhibition of these currents involves cGMP-dependent protein kinase (Sawada *et al*., 1997, Sawada and Ichinose, 1996).

Results of our study on leech Retzius nerve cells demonstrate that sodium nitroprusside in concentrations of 2 mmol/L and 5 mmol/L, induces hyperpolarization. According to results described previously, we can assume that the hyperpolarization, registrated in our study, is probably the result of the NO effect on Ca-activated K^+ channels. It was shown that there were three classes of K channels in soma membrane of leech Retzius nerve cell, the fast and slow Ca-activated channel, and the late calcium regulated potassium channels (Beleslin *et al*., 1988).

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DEJSTVO NATRIJUM NITROPRUSIDA NA MEMBRANSKI MIROVNI POTENCIJAL RETZIUS-OVIH ŽIVČANIH ĆELIJA PIJAVICE

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SADRŽAJ

U ovom radu su izneti rezultati ispitivanja dejstva natrijum nitroprusida (NNP) na membranski mirovni potencijal Retzius-ovih živčanih ćelija (RŽĆ) pijavice (*Haemopis sanguisuga*). Membranski potencijal RŽĆ izolovanih ganglija u Ringerovom rastvoru je registrovan nakon zamene NNP-om (u narednih 30 minuta) i po ponovnom ispiranju Ringerovim rastvorom. Koristili smo rastvor NNP u koncentracijama od 1 mmol/L, 2 mmol/L i 5 mmol/L. Za poređenje pada membranskog potencijala RŽĆ pijavice pri različitim koncentracijala NNP korišćen je

Kruskal-Wallis ANOVA test. Nisu zapažene promene u mirovnom membranskom potencijalu Retzius-ovih nervnih ćelija pijavice korišćenjem koncentracije NNP od 1 mmol/L, dok je koncentracija od 2 mmol/L NNP dovodila do hiperpolarizacije tokom prvih 20 minuta registrovanja. Najveća koncentracija NNP-a od 5 mmol/L dovodila je do hiperpolarizacije tokom prvog minuta registrovanja po zameni rastvora u 50% ćelija, a tokom sledećih 10 minuta u preostalih 50% ćelija. Značajan pad membranskog potencijala je registrovan korišćenjem koncentracije NNP od 5 mmol/L ($p<0.05$).

Hiperpolarizacija RŽĆ izazvana NNP-om (donorom NO) bi mogla da bude posledica dejstva NO na kalijumske kanale RŽĆ pijavice.