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Research Article

# 2-phenyl-1-(3-pyrrolidin-1-il-propyl)-1H-indole hydrochloride (SS-68): Antiarrhythmic and Cardioprotective Activity and Its Molecular Mechanisms of Action (Part II)

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## Abstract

**Introduction.** In previous studies on different animal models, it was shown that compound N-(N-butylpyrrolidine)-2-phenylindole hydrochloride (SS-68) has a broad antiarrhythmic activity. The molecular mechanisms of the pharmacological action of SS-68 were chosen as the focus for this study.

**Materials and methods.** The study of the molecular basis of the pharmacological action of SS-68 was based on 1) molecular docking with the determination of the affinity constant for κ1-opioid receptors; 2) recording the fluorescence of a culture of cardiomyocytes with the determination of the effect of SS-68 on ionic homeostasis; 3) determining the negative chronotropic action in vitro; 4) studying the effect of SS-68 on the transmembrane ion currents of isolated unidentified neurons of the large pond snail (Lymnaea stagnalis), orb snail (Planorbarius corneus) and rat hippocampal neuron cultures.

**Results.** 1) In experiments using molecular docking, the affinity of SS-68 for κ1-opioid receptors is significantly higher than that of butorphanol, but lower than that of (-)-U-50.488; 2) In spontaneously excited preparations of the right atrium, SS-68 causes an irreversible negative chronotropic effect. In experiments on atrial myocardium in rats, SS-68 is capable of demonstrating the ability to block $M_2$ and $M_3$-cholinergic receptors; 3) When studying the effects on cardiac myocyte ion currents, it was shown that SS-68 has moderate $Na^+$, $K^+$ and $Ca^{2+}$ – blocking activity; 4) In the study of isolated neurons, it was shown that SS-68 influences the electrophysiology of neurocytes in a dose-dependent manner.

**Discussion.** The study of the molecular basis of the action of SS-68 showed that this compound has a pleiotropic multitarget effect, which consists of, at least, the effect on $Na^+$, $Ca^{2+}$ and $K^+$-homeostasis of cardiomyocytes and neurons, $M_2$-, $M_3$-cholinergic receptors, and κ1-opioid receptors.

**Conclusion.** From the point of view of molecular pharmacology, SS-68 can be attributed to an antiarrhythmic drug with a mixed type of action.

## Keywords

arrhythmias, antiarrhythmic drugs, SS-68, isolated hippocampal neurons, great pond snail neurons.

# Introduction

Normal heart rhythm is one of the main conditions for maintaining adequate hemodynamics. Cardiac rhythm disorders (arrhythmias) can result from various structural and functional abnormalities, such as myocardial infarction, intoxication, cardiomyopathy, etc. Atrial fibrillation (AF) can be distinguished from among the most common and clinically significant arrhythmias. According to modern concepts, AF is a cardiac rhythm disorder characterized by the absence of atrial systole, which is accompanied by arrhythmic contractions of the ventricles (Revishvili 2015, Camm et al. 2010, Calkins et al. 2009). The proliferation of AF in most countries of the world, as well as the morbidity and mortality due to it have almost doubled (Chugh et al. 2014). In Western Europe, AF is detected in the general population already in 2.0–4.7% of cases (Davis et al. 2012). Over the next 3 decades, the number of patients with AF in Europe and the United States is expected to more than double (Naccarelli et al. 2009, Reardon et al. 2012, Ball et al. 2013, Wilke et al. 2013). At the same time, the proliferation of AF in China is less than half of that of the population of Western countries (Zhou and Hu 2008), among African Americans in the United States it is 45% lower than that of the white population of this country (Jensen et al. 2013).

AF occurs in cases when structural and/or electrophysiological disorders lead to anomalous pulse formation and/or its spread into the atrium tissue. These disorders are due to various pathophysiological mechanisms that have not yet been fully studied, and AF is their final result (Revishvili 2015, Wakili et al. 2011).

In previous studies by the authors of this paper, a high antiarrhythmic potential of SS-68 compound was demonstrated, which was comparable to the main antiarrhythmics, such as amiodarone, etacizin, propranolol, etc, in most of the models on neutralizing and prophylactic activities (Bogus et al. 2018).

In this regard, the molecular mechanisms of the antiarrhythmic action of SS-68 were selected as as the focus for this study.

# Materials and methods

The experiments were carried out in accordance with the requirements of GOST ISO/IEC 1704-2009, GOST R ISO 5725-2002, *The Rules of Laboratory Practice* approved by Order № 708n of the Ministry of Healthcare and Social Development of the Russian Federation of August 23, 2010, and in compliance with *The European Convention for the Protection of Vertebrates Used for Experiments or Other Scientific Purposes* (Directive 2010/63/EU). The experiments were conducted in accordance with *The Guidelines for Pre-clinical Study of Medicinal Products* (2012).

### Effect of SS-68 on $\kappa_1$-opioid receptors

To detect the link of SS-68 with the opioid κ1-receptor, molecular docking to the specific binding site of this SS-68 receptor, butorphanol (a partial $\kappa_1$-receptor agonist) and (-)-U-50,488 (a highly selective $\kappa_1$-receptor agonist) was performed. Docking into the X-ray model of the human κ1-opioid receptor dimer (PDB code 4DJH) (Wu et al. 2012) was performed using AutoDock Vina 1.1.1 package (Trott and Olson 2010) with the calculation of the minimum docking energy ΔE (Vasiliev et al. 2016).

### The study of the negative chronotropic action of SS-68 in the intact atrial and ventricular myocardium and against the background of its cholinergic stimulation in vitro

In the experiments, white nonlinear male rats and male mice were used. For electrophysiological experiments, three types of preparations were isolated: an isolated right atrium operating in its own rhythm, including the atrial appendage, the crista terminalis and the intervenous region with a sinoatrial node; the right atrium, working in the paced rhythm, containing only the atrial appendage (the pacemaker area was removed); ventricular myocardium, which is an isolated wall of the right ventricle. The preparations that did not contain a pacemaker were stimulated through silver electrodes using a linear insulator of stimuli DL-340 (Neuro-biolab, Russia) with a frequency of 5.5 Hz, and the duration of the stimuli was 2 ms.

### The effect of SS-68 on ionic homeostasis of resting and stimulated (intact and ischemic) cardiomyocytes

The isolation of cardiomyocytes from the left ventricle of the white non-linear white male rats, the procedure of loading cells with Fura-2/AM and SBFI probes, the registration of fluorescence were carried out according to the methods described by A. I. Khankoeva et al. (1997, 1998). The effect of SS-68 on the calcium conductivity of the $I_f$/HCN channels of the ventricular cardiomyocytes was studied in experiments on white non-linear male rats. Cardiomyocytes were isolated from the left ventricle of the heart, cells were loaded with a Fura2/AM probe, and fluorescence was recorded according to the method described by A.I. Khankoeva et al. (1997, 1998).

### The effect of SS-68 on $Na^+$-, $Ca^{2+}$- and $K^+$-ionic currents and AP

This was studied in two series of experiments: on isolated and intact unidentified neurons (Kostyuk et al. 1981) of the gastropod mollusk – a great pond snail (Lymnaea stagnalis), orb snail great ramshorn (Planorbarius corneus) and rat hippocampal neuron cultures. In the first series of experiments, the circumesophageal nerve ring was prepared from the mollusc's body, then it was placed in a chamber for recording intracellular potentials with glass microelectrodes. SS-68 and amiodarone were added to external solutions to isolate the corresponding currents. Ion current and potential curves were visually evaluated on an oscilloscope screen, entered into a computer, and printed out. Based on the data obtained by means of the computer, the concentration-effect dependency graphs were made. The initial values of currents and potentials were taken as 100%, and the indicators established under the action of the substances were expressed in % of the initial values.

## Results and discussion

### Effect of SS-68 on κ1-opioid receptors in experiments using molecular docking

In accordance with the calculated binding constants, the SS-68 affinity for κ1-opioid receptors was 4.53 times higher than that of butorphanol, and when compared to (-)U-50.488 – 2.84 times lower (Table 1).

Table 1 shows that the affinity of SS-68 for κ1-opioid receptors is significantly higher than that of butorphanol, but significantly lower than that of (-)-U-50.488.

### Effect of SS-68 on resting and stimulated (intact and ischemic) rat cardiomyocytes on ionic homeostasis

It is known that myocardial ischemia is one of the main causes of arrhythmias (Galenko-Yaroshevsky et al. 2012). Hypoxia-induced shifts of intracellular energy metabolism cause changes in transmembrane ion currents $Na^+$, $K^+$, $Ca^{2+}$, leading to various disorders of automatism and conduction of cardiac cells. On this basis, the effect of SS-68, as well as of amiodarone and dronedarone, taken for comparison, on the ionic homeostasis of resting and stimulated (intact and ischemic) cardiomyocytes was studied.

### Effect on $Na^+$-channels

Under the conditions of our experiments, the diastolic concentration of $[Na^+]_{cyt}$ in resting cardiomyocytes was 7.9±0.5 mM (n=4). Under the conditions of experimental hypoxia induced by KCN and 2-deoxyglucose, a new elevated level of $[Na^+]_{cyt}$ was 16.3±1.5 mM (n=6). Thus, the difference between the basal levels of $Na^+$ in intact and non-oxygenated cardiomyocytes averaged 8.4 mM. Preliminary introduction of SS-68, amiodarone, and dronedarone to a final concentration of 10, 25, and 50 μM caused a dose-dependent decrease in $Na^+$. At the same time, the intensity of the $Na^+$-blocking effect was approximately equal: SS-68, amiodarone and dronedarone, taken at a concentration of 10 μM, significantly ($p<0.05$) inhibited sodium growth by 29, 35 and 27%, respectively. It was possible to differentiate the effects of SS-68, amiodarone, and dronedarone in the experiments on stimulated cardiomyocytes. Electrical stimulation of cells during the entire period of "chemical" hypoxia led to a significant change in $[Na^+]_{cyt}$, both with respect to control samples (ΔNa=15.9±2.3 mM) and in comparison with unstimulated myocytes (ΔNa=7.5±1.8 mM). The rise of $[Na^+]_{cyt}$ did not depend on the chosen frequency of electrical discharges: stimulation of cells with a current of 0.2 or 1.0 Hz caused a similar increase in the level of $Na^+$. The inhibitory effect of amiodarone on an increase in $[Na^+]_{cyt}$ depended not only on the dose, but also on the frequency of electrical stimulation of cardiomyocytes (Fig. 1). It should be noted that the depth of the $Na^+$-blocking effect of amiodarone on the stimulated cells was significantly greater than on the unstimulated ones in the whole range of concentrations. Dronedarone inhibited the induced $[Na^+]_{cyt}$ to a lesser extent, and the dependence of the activity of this drug on stimulation with a current of various frequencies was also detected (Fig. 1). From the data presented in Figure 1, it is clear that amiodarone-like effect on $[Na^+]_{cyt}$ is characteristic of SS-68. Depending on the stimulation frequency imposed, the degree of inhibition of sodium current by SS-68 was significantly different.

### Effect on $Ca^{2+}$-channels

In well-oxygenated cardiomyocytes, the diastolic concentration of free $Ca^{2+}$ ions in the sarcoplasm is maintained at the level of 125–145 nM.

The study of the effects of SS-68, amiodarone, and dronedarone on the content of $Ca^{2+}$ in resting cardiomyocytes did not reveal a significant change in the $Ca^{2+}$ res-

**Table 1.** Results of docking SS-68, butorphanol and (-)-U-50,488 substance into the specific κ1-opioid receptor binding site

| Substance | Docking energy ΔE, kcal/mol | Binding constant K, nM |
|---|---|---|
| SS-68 | -8.60 | 538.10 |
| Butorphanol | -7.70 | 2437.30 |
| (-)-U-50,488 | -9.20 | 196.50 |

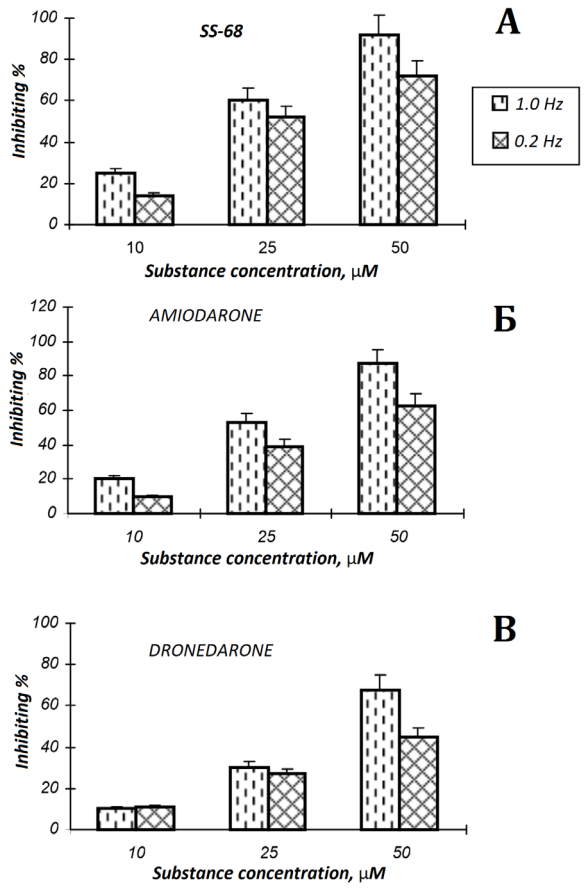


**Figure 1.** Comparative effectiveness of the $Na^+$-blocking effect of SS-68 (a), amiodarone (b) and dronedarone (c) under conditions of experimental hypoxia.

ponse of cells within the concentration range of 10–100 μM. Thus, the obtained data suggest that the substances under study do not change the compartmentalization of intracellular $Ca^{2+}$ in unstimulated intact cardiomyocytes. During experimental hypoxia induced by incubation of cells with D-glucose and KCN for 20 minutes, significant

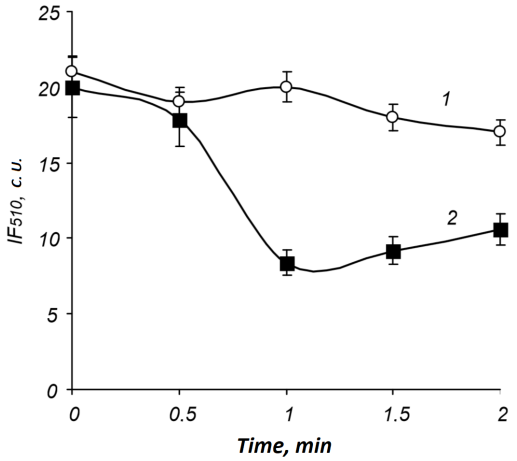


**Figure 2.** Fluorescence (510 nm) of resting (1) and stimulated (2) (with electrical impulses of 1.0 Hz) cardiomyocytes.

changes in the $Ca^{2+}$ exchange of cardiomyocytes were observed. Under the conditions of "chemical hypoxia", a certain diastolic level of $Ca^{2+}$ was higher than in intact cells (ΔCa=62 nM).

### Impact on $K^+$-channels

The limitations when regitering $K^+$-current registration using the fluorescent $K^+$ PBFI ion indicator made it possible to evaluate only qualitative changes in the intracellular content of $[K^+]_{cyt}$. Electrical stimulation of cardiomyocytes was accompanied by a noticeable decrease in probe fluorescence intensity recorded for 2 minutes (Fig. 2).

SS-68, amiodarone and dronedarone influenced both the absolute values of fluorescence and the dynamics of changes in the PBFI fluorescence (Fig. 3). Depending on the severity of the $K^+$-blocking properties, the test substances can be arranged in a row as follows: SS-68>dronedarone>amiodarone. Thus, SS-68 and, taken as reference

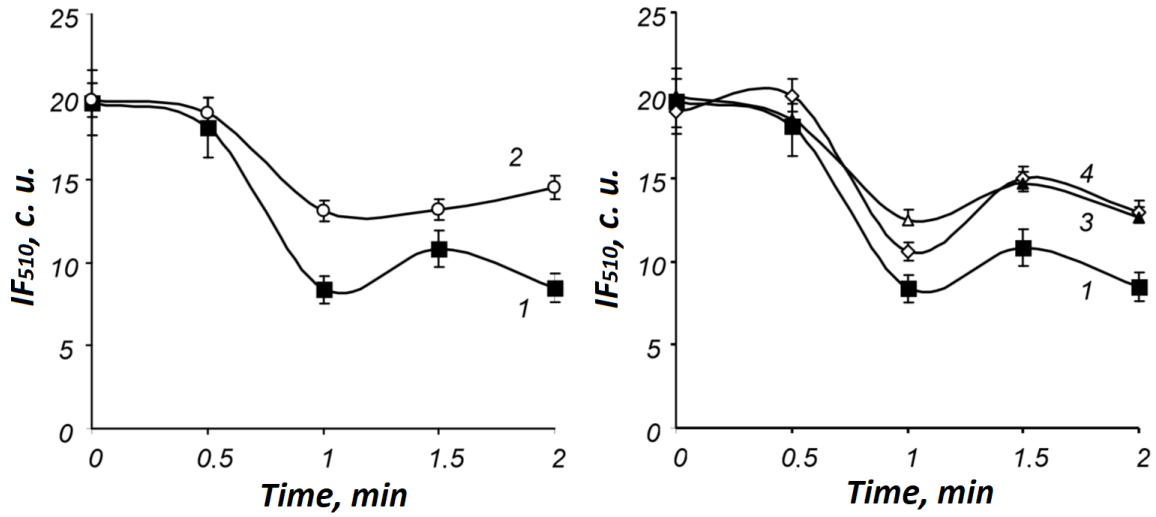


**Figure 3.** Dynamic pattern of PBFI probe fluorescence of the during electrical stimulation of cardiomyocytes without SS-68 (1) and with SS-68 (2), dronedarone (3) and amiodarone (4), taken at a concentration of 50 μM.

drugs, amiodarone and dronedarone under conditions of unstimulated cardiomyocytes (both intact and ischemic) cause an almost equivalent dose-dependent decrease in their $[Na^+]_{cyt}$ concentration.

SS-68 has an indirect effect on the $Ca^{2+}$ homeostasis of cardiomyocytes by blocking the entry of $Na^+$ into the cell. In contrast to the effect of SS-68, the effect of amiodarone and dronedarone on $[Ca^{2+}]_{cyt}$ in cardiomyocytes is a combination of its direct effect on the $Ca^{2+}$ exchange and its indirect action, realized through the mechanism of inhibiting Na-dependent elevation of $[Ca^{2+}]_{cyt}$. In terms of its ability to have a $K^+$-blocking effect, SS-68 is superior to dronedarone and amiodarone.

**Study of the negative chronotropic action of SS-68 in the intact atrial and ventricular myocardium and against the background of its cholinergic stimulation in vitro**

The parameters of electrical activity most sensitive to the action of SS-68 were $APD_{50}$ (action potential duration at 50%) and $APD_{90}$ (action potential duration at 90%). Experiments showed that, unlike all other concentrations, the smallest of the used concentration cuased not an increase of APD, but, on the contrary, a small shortening of AP, which was statistically significant both in ventricular myocardial preparations (Figs. 4 A, 6 A) and in the atria, working in their own rhythm (Fig. 5, 7). In the atria, which worked in the paced rhythm, a significant decrease in APD was observed only for 50% of the repolarization (Figs. 4 E, 6 C). However, in the atrial myocardium, when washing-off from $10^{-6}$ M of SS-68, instead of the expected gradual return of APD to the control values, an increase in APD was observed compared to control, which was then not eliminated even after 20 min of washing-off (Figs. 6 D, 7 B). In the ventricular myocardium, this phenomenon was not observed. In the other concentrations, SS-68 caused the same type of effects – an increase in $APD_{90}$ and $APD_{50}$, which showed pronounced dose-dependence (Figs. 6 B and D, 7 F-H, 5 B-D, 6 A and B, 7 A). At the same time, during washing-off, these effects were always significantly enhanced (Figs. 6 B and D, 7 B). For exam-

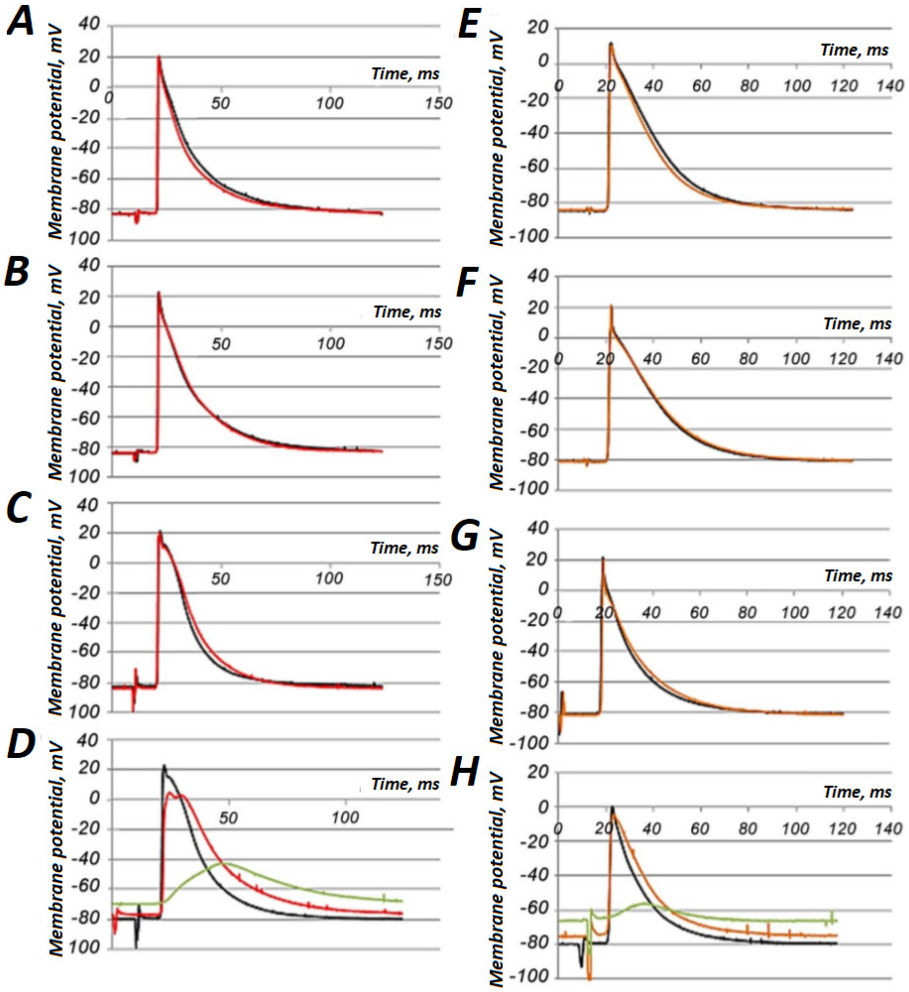


**Figure 4.** Original records of right ventricular myocardial AP (A–D), as well as of the right atrium operating in a paced rhythm, (E–H) in normal conditions and when applying SS-68 at concentrations of $10^{-6}$ (A), $5\times10^{-6}$ (B), $10^{-5}$ (C) and $5\times10^{-5}$ M (D).
**Key.** The control record is shown in black line, the record at the time of the maximum effect of SS-68 is red (ventricular myocardium) or orange (atrial myocardium). In Figures D and H green lines show the records of electrical activity during the washing off the drug. Records are taken from two representative experiments.

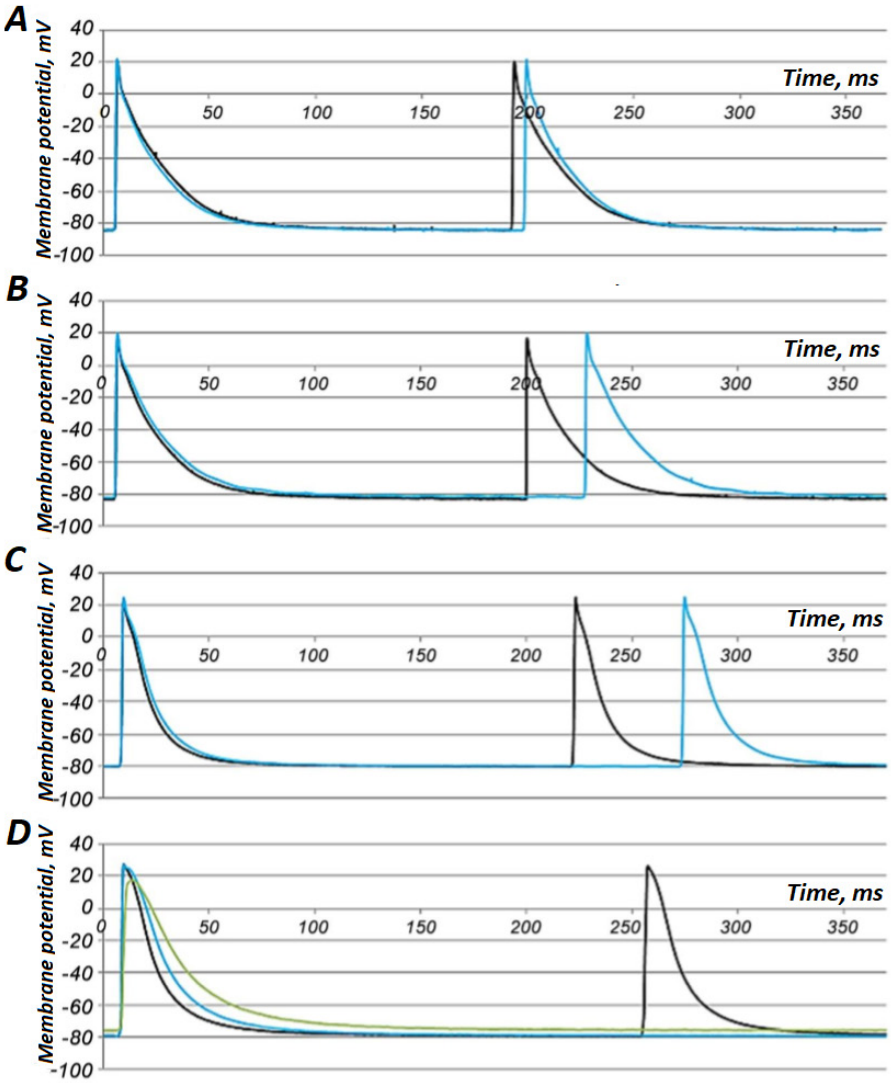


**Figure 5.** Original recordings of right atrial AP, working in its own rhythm, in normal condition and when applying SS-68 at concentrations of $10^{-6}$ (A), $5\times10^{-6}$ (B), $10^{-5}$ (C) and $5\times10^{-5}$ M (D).

**Key.** The control record is shown in black line, the record at the time of the maximum effect of SS-68 is blue. In Figure D, green indicates the record of electrical activity during the washing off the drug (at the moment before withdrawal of the drug). Records are taken from one representative experiment.

ple, if in the ventricular myocardium and in the atria that worked in their own rhythm, $5\times10^{-6}$ M of SS-68 did not cause a significant lengthening of AP, then after washing off the substance in such a concentration, a marked increase in $APD_{50}$ and $APD_{90}$ was observed (Figs. 6 B, 7 B).

The most pronounced effects developed under the influence of the highest concentration used ($5\times10^{-5}$ M), and especially during washing-off from it. In addition to the pronounced extension of AP, SS-68 in this concentration led to a sharp decrease in the amplitude of AP in preparations that worked in the paced rhythm (by 27.6±4.3% in the ventricular myocardium, by 24±4.4% in the atrial myocardium), linked with a decrease in membrane potential (by 5.2±0.8 mV and 4.4±0.9 mV, respectively). During washing-off, in 5 ventricular preparations and in 4 out of 6 atrial preparations working in the paced rhythm, complete inhibition of electrical activity happened – instead of AP, myocardial cells generated only an electrotonic response (Figs. 4 D and H).

### The effect of SS-68 on $Na^+$, $Ca^{2+}$ and $K^+$ transmembrane ion currents and the action potential of isolated unidentified gastropod neurons and rat hippocampal neuron cultures

It was found out that under the influence of SS-68 and amiodarone, used as a reference drug, at concentrations of 1 and 10 μM, the amplitude of the sodium current changed slightly, while SS-68 increased slightly (by 3–5% of control), and amiodarone did not change or slightly reduced its amplitude (Fig. 8 A). At concentrations of 100 and 1000 μM, current-dependent inhibition of current occurred, while SS-68 inhibited it to a greater extent than amiodarone. The recovery of current after the action of SS-68

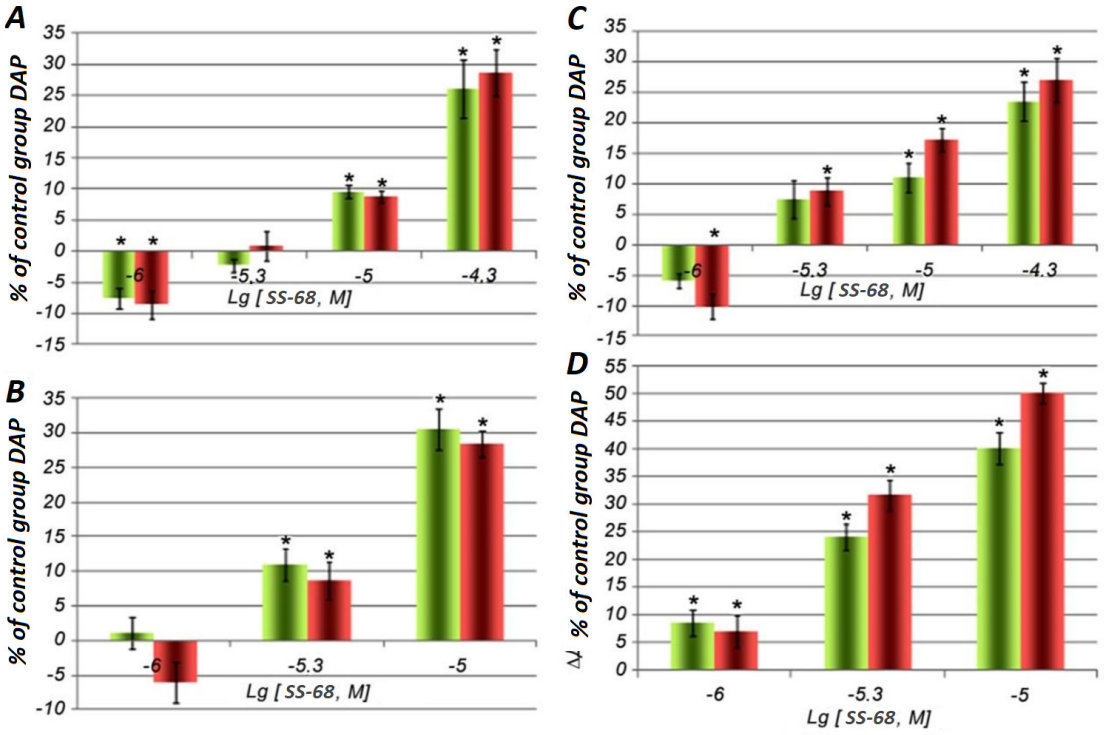


**Figure 6**. Dose-dependence of APD changes under the influence of SS-68 (A, C) and during washing-off (B, D) in preparations of the right ventricular wall (A, B) and preparations of the right atrium, working in a paced rhythm (C, D).
**Key.** Bar charts: green – $APD_{90}$ changes, red – $APD_{50}$. * Reliability of effect ($p<0.05$, Wilcoxon test).

was very slow (over 15 minutes), and after amiodarone – faster (within 5–7 minutes up to 70% of control), which indicates the greater binding strength of SS-68 molecules to membrane structures (or ion channels) compared to amiodarone.

The nature of the change in sodium currents under the influence of SS-68 is shown in Figure 8 B (decrease in amplitude). The peak of current-voltage characteristics of the membrane for sodium channels under the influence of SS-68 slightly (up to 5 mV) shifted to the right (towards the depolarization of the membrane) along the potential axis (Fig. 8 C), which indicates a change in the potential of the surface charge of the membrane created by fixed charges. The kinetics of sodium current development under the influence of amiodarone did not change, and there was no shift in the maximum current-voltage characteristic of the membrane. When registering the current-voltage characteristic of the membrane for sodium and potassium channels, the corresponding currents were simultaneously inhibited to approximately the same degree (Fig. 8 D).

Under the influence of amiodarone and SS-68, the calcium current amplitude only decreased dose-dependently and reversibly, while amiodarone inhibited it to a lesser extent (Fig. 8 A). The reversibility of the inhibition effects after the washing-off almost reached the original values, with amiodarone being washed off 2–3 times faster (in 5–7 minutes) than SS-68 (in 15–25 minutes). The kinetics of the development of calcium current under the influence of amiodarone was slightly accelerated (Fig. 8 C), but did not change under the influence of SS-68 (Fig. 8 B). Under

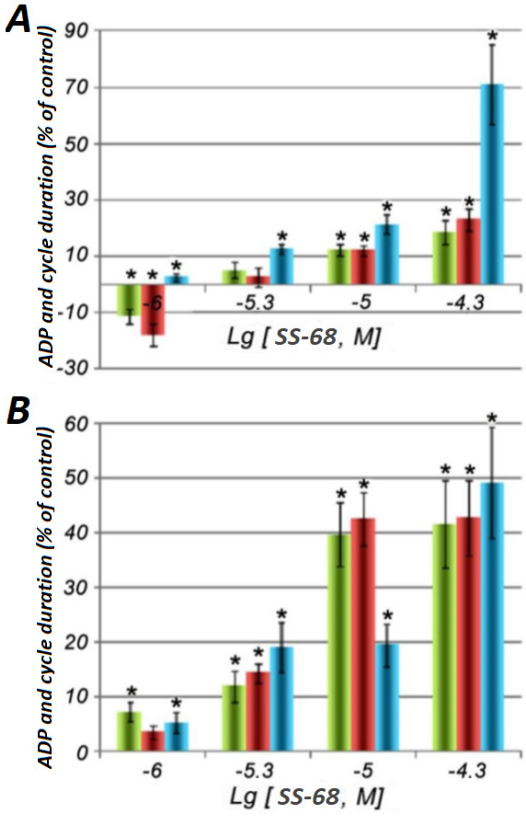


**Figure 7**. Dose-dependent changes in APD and duration cycle under the influence of SS-68 (A) and during washing-off (B) in right atrial preparations, working in their own rhythm.
**Key.** Bar charts: green – $APD_{90}$ changes, red – $APD_{50}$, blue – duration cycle. * - Reliability of effect ($p<0.05$, Wilcoxon test).

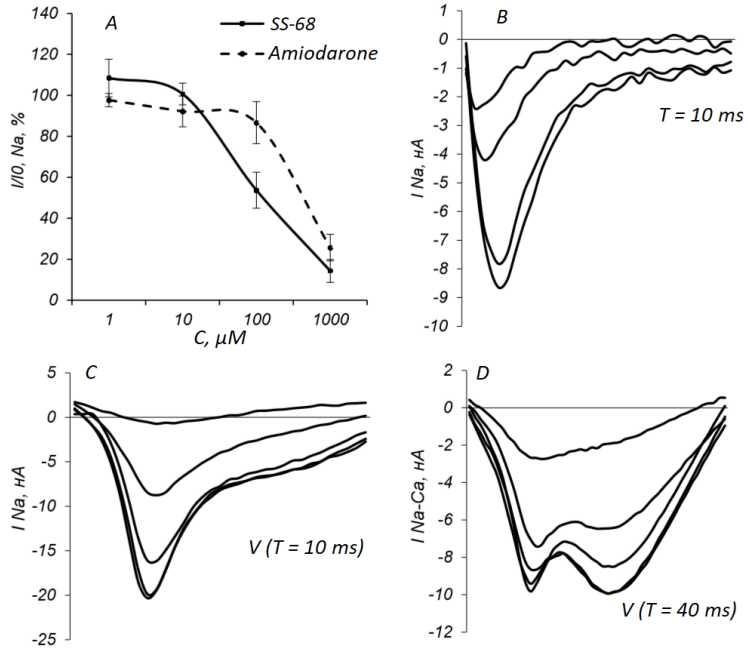


**Figure 8**. Changes in the sodium current of the pond snail neurons under the influence of SS-68 (n=6) and amiodarone (n=6).
**Key.** A – concentration-effect relations under the influence of SS-68 and amiodarone. B – changes in the amplitude and kinetics of the current under the influence of, SS-68, curves from bottom to top: 1 – 1 μM, 2 – control, 3 – SS-68 100 μM, 4 – 1000 μM. B – current-voltage characteristics of sodium channels, from bottom to top: 1 – control, 2 – 1 μM, 3 – 10, 4 – 100, 5 – 1000 μM. G – the same for Na-calcium channels: 1 – control, 2 – 1 μM, 3 – 10, 4 – 100, 5 – 1000. On the x-axis: A – concentration, B – time, C and D – sawtooth offset of membrane potential from –40 to 50 mV for 10 and 40 ms; on the ordinate axis – ion current (A: I – under the influence of the substance, I0 - before the influence; confidence intervals at p=95%; B and C: $I_{Na}$ - sodium current).

the influence of both substances, the maximum of the current-voltage characteristics did not shift along the potential axis (Fig. 9 D), i.e. the potential of the fixed charges of the membrane did not change. The nature of the effects of amiodarone and SS-68 on slow potassium channels (Fig. 8 A) resembled the effect on sodium channels, and for SS-68 it was two-phase; at a concentration of 1 μM, the amplitude of the current increased slightly, and at higher levels it decreased (at a concentration of 1000 μM – by 15–20%), under the influence of amiodarone, the changes were monophasic (inhibition). In general, the inhibition of current by SS-68 was stronger than by amiodarone.

The restoration of the currents in the process of washing off neurons was similar for calcium and sodium currents – more slowly for SS-68. Under the influence of both substances at concentrations of 100 and 1000 μM, inactivation of the potassium current accelerated. There were no shifts of the current-voltage characteristics of the channels, that is, the potential of the fixed charges of the membrane near the potassium channels did not change. The nature of the effect of SS-68 and amiodarone on fast potassium currents outwardly resembled their effect on sodium (monophase), there were no changes in the kinetics of their development (Figs. 10 C and D in the left part of the figures). For comparison, the general nature of inhibiting incoming sodium and calcium ion currents, as well as fast and slow potassium ion currents under the influence of SS-68 is shown in Figure 11 A. At the very end of the recording, the capacitance currents of the membrane poining downward and arising in response to shutdown of the linearly increasing offset potential.

In a small series of experiments on 4 neurons of a pond snail, a slow potassium current was recorded, while SS-68 at a concentration of 100 μM was supplied not from the outer part of the neuron membrane, but was injected inside with dialysis fluid, and its effect was tested from inside the cell. A typical reaction of one of the neurons is shown in Figure 11 B. It turned out that SS-68, inhibiting current in the same concentration from the outside (Fig. 11B, 4$^{th}$ curve from top under the arrow), did not inhibit current from inside (Fig. 11 B, the two upper curves – control and intracellular action, the third curve from top – washing SS-68 off from the outside). It can be assumed that potassium channels are available for binding to SS-68 only in case of extracellular action: either through membrane lipids, or after entering the channel from the outside when it is opened. Similar reactions were recorded for sodium and calcium currents. To control and compare the intracellular action of SS-68 with that of other substances under similar conditions, the effect of lidocaine on potassium ion channels from inside and outside the cell at a concentration of 1000 μM was tested. It was shown that lidocaine in intracellular action, as compared with extracellular action, turned out to be ineffective. Washing lidocaine off from outside led to a gradual recovery of current. It is known that tetraethylammonium (TEA) ef-

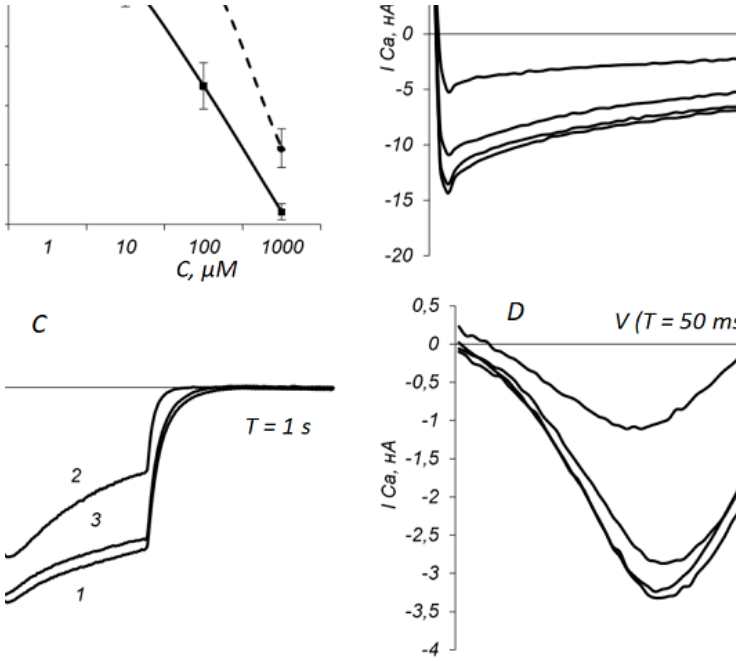


**Figure 9**. Changes in the calcium current of the pond snail neurons under the influence of SS-68 (n=7) and amiodarone (n=6).
**Kew.** A – concentration-effect relations. B – changes in the amplitude and kinetics of the current under the influence of SS-68, curves from bottom to top: 1 – control, 2 – 10 μM, 3 – 100, 4 – 1000. C –changes in the amplitude and kinetics of the current under the influence of amiodarone; curves from bottom to top: 1 – control, 2 – laundering, 3 – 100 μM. D – current-voltage characteristics under the influence of SS-68, curves from bottom to top: 1 – control, 2 – 10 μM, 3 – 100, 4 – 1000. On the x-axis: A – concentration of anesthetics, B and C – time, D – sawtooth offset of membrane potential from –40 to 50 mV; on the ordinate axis – ion current (A: I – under the influence of the substance, $I_0$ – before the influence; confidence intervals at p=95%; $I_{Ca}$ – calcium current).

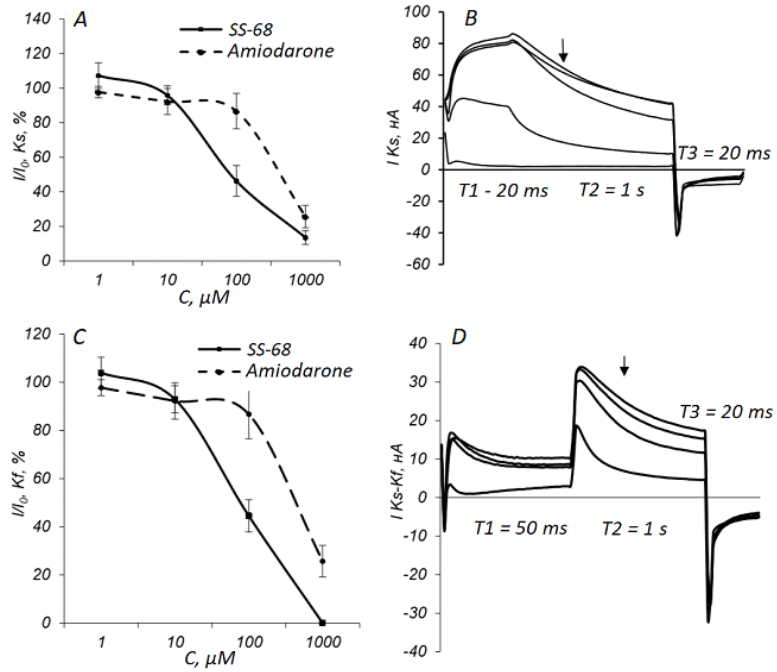


**Figure 10.** Changes in potassium slow and fast currents of pond snail neurons under the influence of SS-68 (n=6 and n=5, respectively) and amiodarone (n=6 and n=5).
**Key.** A and B – concentration-effect relations for slow and fast potassium channels. B – changes in the amplitude and kinetics of current; curves from top to bottom under the arrow: 1 – 1 μM, 2 – control, 3 – 10 μM, 4 – 100 μM, 5 – 1000 μM. C - current-voltage characteristics: 1 – control, 2 – washing-off, 3 – SS-68 100 μM, 4 – SS-68 1000 μM. D is the same as B; curves under the arrow: 1 – control, 2 – 10 μM, 3 – 100, 4 – 1000 μM. On the x-axis: A and C – concentration, B and D – time; on the ordinate axis – ion current (A and B: I – under the influence of the substance, I0 – before the influence; confidence intervals at p=95%; B and D: I Ks – slow and I Kf – fast potassium currents).

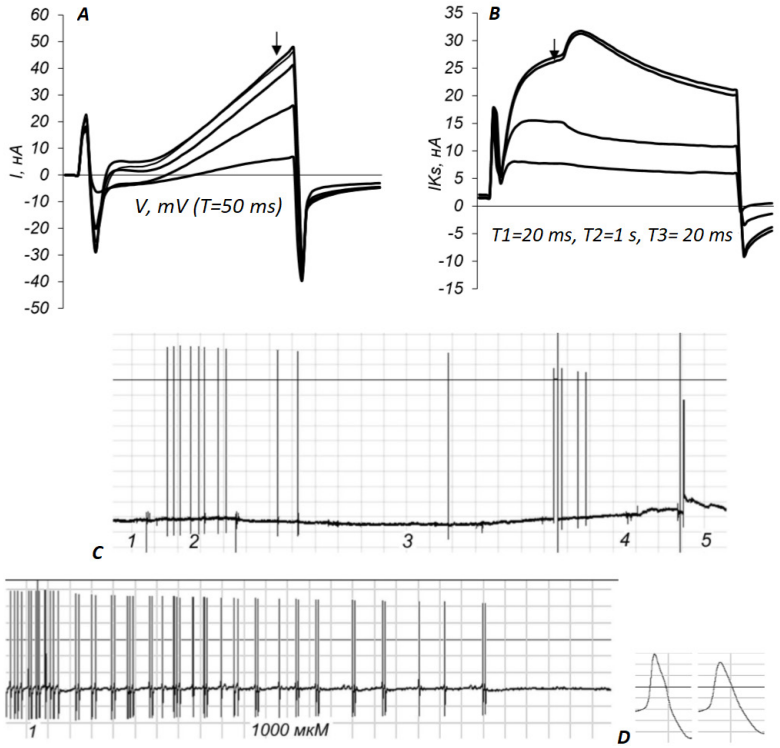


**Figure 11**. Changes in ion currents and intracellular potentials of neurons of the pond snail and great ramshorn under the influence of SS-68.

**Key.** A – current-voltage characteristics of the membrane, under the arrow from top to bottom: 1 – 1 μM SS-68, 2 – control, 3 – SS-68 10 μM, 4 – 100, 5 – 1000; B – changes in the amplitude and kinetics of potassium currents during extra- and intracellular action; curves from top to bottom under the arrow: 1 – control, 2 – 100 μM шт case of intracellular action, 3 – outside washing-off, 5 – 100 μM outside; C – the dynamics of changes in the resting potential and the electrical activity of the neuron of the orb snail under the influence of SS-68 in various concentrations: 1 – control, 2 – 1 μM, 3 – 10, 4 – 100, 5 – 1000. On the x-axis: time, one cell=1 min; On the ordinate axis - membrane potential, one cell=5 mV; D – the same as C: 1 – at the beginning of the action of 100 μM, then – of 1000 μM and cessation of activity (on the x-axis: time, one cell=1 min; on the ordinate axis – membrane potential, one cell=15 mV) ; E – AP parameters: at 100 and 1000 μM (on the x-axis: time, one cell=20 ms; on the ordinate axis – membrane potential, one cell=15 mV); A and B: on the x-axis A – sawtooth offset of membrane potential from –30 to 50 mV in 50 ms, B – time; on the ordinate axis – ion current.

fectively inhibits potassium ion currents in case of external and intracellular actions. Indeed, TEA at a concentration of 10 mM from the inside inhibited by about half the initial part of the current associated with fast potassium channels, and the current of slow potassium channels – by about 25%. The action was reversible, but rather long (after 13 min – current was partially recovered, and after 30 min – current was recovered more completely).

Under the influence of SS-68 at concentrations of 1, 10 and 100 μM, a slight dose-dependent hyperpolarization of 1-3 mV was observed in neurons, whereas when using this substance at a concentration of 1000 μM there was only minor depolarization (Fig. 11 C, fragment 3). Against the background of hyperpolarization, a decrease in the impulse activity of neurons occurred. With the development of cell depolarization, there was a slight increase in impulses in strings of APs with a decrease in the AP amplitude and with a slight increase in their duration. It should be noted that these are phenomena of the usual potential-dependent changes in the parameters of AP. But since the inhibition of ion currents occurs at SS-68 concentrations of 100 and 1000 μM, changes in the parameters of AP can be additionally determined by this circumstance. The decrease in the AP amplitude is clearly shown in Figures 11 D and E – up to the cessation of their generation. However, AP could be caused by passing a depolarizing current through a microelectrode.

## Effect of SS-68 on ionic currents and the action potential of rat hippocampal neuron cultures

### *The effect of SS-68 on induced AP in the hippocampal neurons*

Figures 12 and 13 show the records of changes in the potential of neurons that generate, in control, strings of 13–11 APs with a frequency of 35–15 Hz. It was shown that in all cases in the presence of 1–3 μM of SS-68, the frequency of APs in the string decreased and the duration of individual APs increased due to inhibition of the acti-

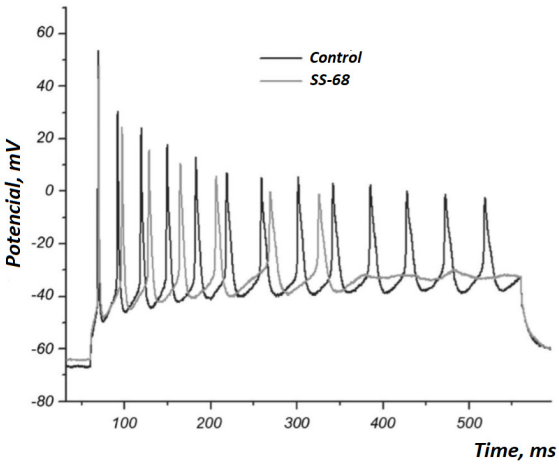


**Figure 12**. Depolarization-induced (300 pA, lasting for 500 ms) AP series in neuron. Under the influence of 3 μM of SS-68, the frequency decreases from 35 to 17 Hz. The culture age is 15 days.

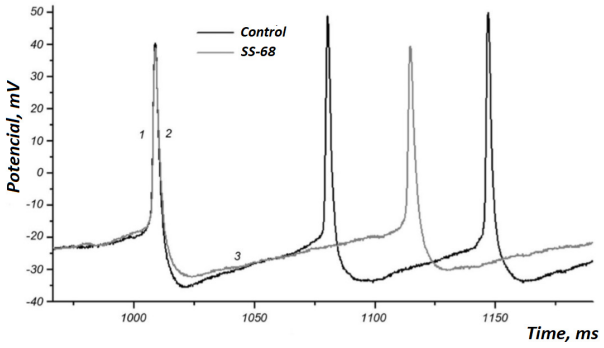


**Figure 13**. The first 3 APs out of 10 generated by neuron 2 in response to the depolarizing impulse. In the presence of 1–3 μM of SS-68, the duration of APs increases due to a decrease in hyperpolarization and inhibition of the slow phase of depolarization.

vity of $K^+$-channels participating in the second phase of repolarization and the third phase of slow depolarization (trace hyperpolarization). The number of APs in the string is reduced due to the rapid vibration reduction. Figure 14 shows the induced single APs of the neuron before and after the application of SS-68. It shows that even at a concentration of 5 μM, SS-68 inhibits the first phase of rapid depolarization, which indicates a partial blockade of $Na^+$-channels. This leads to an increase in the excitability threshold and inhibition of the nerve conduction velocity. There is also a strong inhibition of $K^+$ channels determining the speed of the repolarization phase.

### *The effect of SS-68 on ion currents in the mode of fixing the potential on the neuron membrane*

To determine the type of ion currents, the inhibition of which leads to the observed changes in AP, measurements were performed in the mode of fixing the potential. When registering the current to measure the activity of

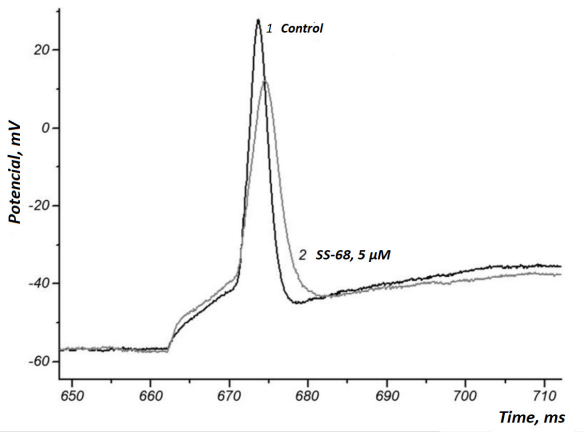


**Figure 14**. In response to a depolarizing impulse of 300 pA lasting for 50 ms, the rat hippocampal neuron generates one AP in the control (1) and in the presence of 5 μM of SS-68 (2).

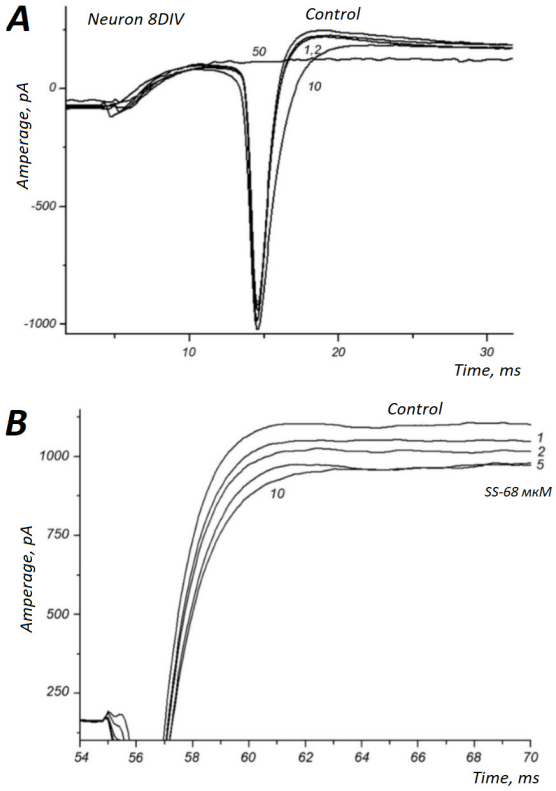


**Figure 15.** A) – Change in $K^+$-current in a neuron in response to depolarization down to -30 mV in control and in the presence of 1, 2, 10 and 50 μM of SS-68. The culture age is 8 days. B) -Change of $K^+$-current in the same neuron in response to depolarization up to +50 mV in the control and in the presence of 1, 2, 5, and 10 μM of SS-68. The culture age is 8 days.

$Na^+$-channels, the cell was depolarized to -30mV (Fig. 15A), then to register the activity of $K^+$-channels, the depolarization was increased to +50mV (Fig. 15B). The measurements were carried out in the presence of various concentrations of SS-68. Figures 15 A and B show that

in low concentrations (1–5 μM), SS-68 inhibits the fast and slow components of $K^+$-current of delayed rectification. As the concentration increases, the $Na^+$-current is also inhibited. When the concentration is increased to 50 μM, SS-68 completely blocks the $Na^+$-potential-dependent channels and the generation of sodium current. Thus, SS-68 in low concentrations selectively blocks repolarization-mediating $K^+$-currents of delayed rectification of neurons, which leads to prolongation of APs, a decrease in the generation rate AP pulses during neuron string activity, and partially blocks $Na^+$-channels, somewhat inhibiting the very first phase of rapid depolarization.

# Discussion

### Effect on opioid receptors

Based on the results presented in Part 1 of the article, it can be assumed that SS-68 in low doses has properties of class III antiarrhythmic and in relatively large doses – those of II, III and IVclasses according to the Vaughan-Williams classification. At the stage of studying antiarrhythmic activity, it was suggested that SS-68 could also have an agonistic effect on opioid receptors. Further, this served as the basis for the study of SS-68 in this direction.

In molecular docking experiments, the affinity of SS-68 for κ1-opioid receptors is significantly higher than that of butorphanol, but lower than that of (-)-U-50.488. Therefore, it can be argued with a certain probability that the antiarrhythmic efficacy of SS-68 in low doses (20 and partially 50 μg/kg) in heart rhythm disturbance conditions against the background of vagus nerve stimulation is realized through an agonistic effect on κ1-opioid receptors.

***In the study of negative chronotropic action*** in vitro, it was found that SS-68 causes two types of effects similar in the atrial and ventricular myocardium of rats: reversible acceleration of the AP repolarization phase and a more pronounced irreversible slowing of the repolarization along with a decrease in the amplitude of AP and resting potential. When using SS-68 in increasing concentrations, the effects of the second type mask the acceleration of repolarization.

In the spontaneously excited preparations of the right atrium, SS-68 causes an irreversible negative chronotropic effect, which is partly a consequence of the development of the irreversible effects of SS-68 in the working myocardium. The mechanism of action of SS-68 appears to include a complex effect on a range of ion currents, in particular, inhibition of $I_K$ and direct inhibition of $I_{Na}$.

SS-68 in the concentration range of $5\times10^{-6}$-$5\times10^{-5}$ M induces in the SAN preparations of mice a dose-dependent slowdown of the rhythm induced by a decrease in the speed of slow diastolic depolarization, as well as a decrease in the rate of rising slope steepness of the action potential. The high concentration ($5\times10^{-5}$ M) of SS-68 entails a decrease in the excitability of the cells of the central part of the SAN and the irregularity of the electrical activity.

In the working atrial myocardium on the background of cholinergic stimulation, $10^{-5}$ M of SS-68 causes a bigger increase in the duration of AP, but less pronounced slowing of the sinus rhythm than in normal conditions. Against the background of $10^{-5}$ M of SS-68, carbachol ($10^{-7}$ M) induces effects that are somewhat weaker than in normal conditions.

In experiments on the atrial myocardium of rats, SS-68 is able to reduce both the effect of joint activation of $M_2$- and $M_3$- cholinergic receptors and the effect of selective stimulation of $M_3$- cholinergic receptors.

***When studying the effect of SS-68 on the content of $Na^+$, $Ca^{2+}$ and $K^+$ in cardiomyocytes***, it was found that in conditions of unstimulated intact and ischemic cardiomyocytes of rats, SS-68, amiodarone and dronedarone, used as reference drugs, cause an almost similar dose-dependent decrease in their $[Na^+]_{cyt}$ concentration, and in stimulated ones – have an inhibitory effect on the increase in $[Na^+]_{cyt}$, both depending on the dose and on the frequency of their electrical stimulation, and the intensity of the $Na^+$-blocking effect of amiodarone is significantly greater than on unstimulated cardiomyocytes; SS-68 exhibits amiodarone-like effect; Dronedarone inhibits $[Na^+]_{cyt}$ to a lesser extent and, like amiodarone and SS-68, is more active on stimulated cardiomyocytes.

SS-68 has an indirect effect on the $Ca^{2+}$ homeostasis of cardiomyocytes by blocking the entry of $Na^+$ into the cell. Unlike the effect of SS-68, the effect of amiodarone and dronedarone on $[Ca^{2+}]_{cyt}$ in cardiomyocytes consists of its direct effect on the $Ca^{2+}$ exchange and the indirect effect realized through the mechanism of inhibiting the Na-dependent rise in $[Ca^{2+}]_{cyt}$.

By ability to exert a blocking effect on the $K^+$ homeostasis in cardiomyocytes, SS-68 is superior to amiodarone and dronedarone.

SS-68 and ivabradine, used as a reference drug, in experiments on resting and stimulated cardiomyocytes of rats almost similarly inhibit the transport of $Ca^{2+}$ through $I_f$/HCN channels. The change of $Ca^{2+}$ diastolic current under the influence of SS-68 through these channels is direct. One of the pathogenetic mechanisms of atrial arrhythmogenesis can be the conduction of $Ca^{2+}$ current through the $I_f$ channel, confirmed by activation of the cAMP channel and inhibition by the $I_f$-channel blocker with ivabradine and SS-68.

The experimental approach to determining the conductivity of $Ca^{2+}$ through $I_f$/HCN channels proposed in the present paper can be used in further studies of the pathogenetic mechanisms of atrial arrhythmias and their possible pharmacological correction. The presence of $Ca^{2+}$-current in the $I_f$/HCN channel, as a universal cell messenger, is important for understanding the mechanisms of development of cardiovascular diseases, as well as in studies of the arrhythmogenic properties of the $I_f$.

SS-68 and amiodarone have a pronounced ***membranotropic effect***, which is manifested in changes in ion currents

(***through the potential-controlled ion channels of the isolated neurons of the pond snail***) and their membrane potentials. In low concentrations (1 and 10 μM), SS-68 causes activation of $Na^+$ and $K^+$ slow currents, acceleration of inactivation of $K^+$-currents and slight hyperpolarization of neurons; in high (100 and 1000 μM) induces dose-dependent and approximately equivalent inhibition of all investigated ion currents which was manifested to a greater extent than in amiodarone; the latter, compared with SS-68, inhibits ion currents to a lesser extent, but it is washed off faster.

In experiments on isolated hippocampal neurons of rats, SS-68 at low concentrations (up to 2-3 μM) selectively blocks the repolarization-mediating $K^+$-currents of delayed rectification, which leads to prolongation of action potential, a decrease in the AP generation rate during neuron string activity, and in high concentrations it partially blocks $Na^+$-channels, somewhat inhibiting the first phase of rapid depolarization.

## Conclusions

Thus, SS-68 compound is a promising pharmacological agent with a high preventive and arresting effects towards various electrophysiological disorders in the heart. From the point of view of molecular pharmacology, SS-68 can be referred to an antiarrhythmic drug with a mixed type of action.

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# Author contributions


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