Elucidation of the Mechanisms of Membranotropic Effects of RU-1203 on Ionic Channels of Lymnaea Stagnalis Neurons

O. Yu. Grechko, A. A. Spasov, A. I. Vislobokov*, Yu. D. Ignatov*, and V. A. Anisimova**

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RU-1203-induced norBNI-irreversible inhibition of sodium (INa), calcium (ICa), and slow and fast potassium currents (IKs and IKf) was demonstrated in isolated neurons of *Lymnaea stagnalis*.

Key Words: *κ-opioid receptors; ionic channels; Lymnaea stagnalis neurons; benzimidazoles; norbinaltorphimine*

Pronounced membranotropic effect of RU-1203, a new condensed benzimidazole derivative, on isolated neurons of *Lymnaea stagnalis* was demonstrated in experiments using intracellular dialysis and patch-clamp technique; the effect was similar to that produced by anesthetics [1]. *In vitro* experiments on rabbit ductus deferens exclusively expressing a subpopulation of κ-opioid receptors revealed analgesic properties of this compound determined by κ-receptor nature of its effect [6].

According to published data, receptor-mediated mechanisms play the key role in the realization of the effects of opioid analgesics. κ-Selective agonists norBNI-reversibly modulate functional activity of calcium, potassium, and indirectly sodium potential-dependent channels due to primary interaction with receptors through intracellular signaling systems [7,9, 10,13]. At the same time, principally new possible effects of κ-selective ligands on sodium, calcium, and potassium ionic currents in neuronal membrane via their interaction with channel structures were demonstrated [11,15]. It is believed that κ-receptor agonists can decrease the number of functioning currents, bind gating components of the channels, and reduce ionic channels by shortening the time of open state of individual channels or reducing the frequency of their opening [1].

Here we studied the mechanisms of membranotropic effects of κ-receptor agonist RU-1203 on ionic channels of *Lymnaea stagnalis* neurons.

MATERIALS AND METHODS

Patch-clamp technique and intracellular dialysis were used [1]. The isolated cell was placed on a polyethylene pipette (in most cases at holding potential of -90 mV). The neuronal membrane within the pipette mouth was destroyed by negative hydrostatic pulses thus allowing electrical contact of inner cell media with non-polarizing electrode connected with patch-clamp amplifier.

RU-1203 (2-fluorophenyl-9-pyrrolidinethylimidazo[1,2-a]benzimidazole) was tested in a concentration of 500 μM. The specificity of κ-opioid nature of RU-1203 action was confirmed in a special series of tests with κ-selective antagonist norbinaltorphimine (norBNI; 17.17'-(dicyclopropylmethyl)-6,6'-imino-7,7'-binorphinan-3.4',14.14'-tetrol; Sigma) in concentrations of 0.1, 1, 5, and 10 μM.

After recording total ionic currents, the intra- and extracellular solutions were replaced with solutions for recording individual currents [1]. Pure calcium

Volgograd State Medical University; *St. Petersburg State I. P. Pavlov Medical University, Federal Agency for Health Care and Social Development of the Russian Federation; **Research Institute of Physical and Organic Chemistry, South Federal University, Rostov-on-Don, Russia. ***Address for correspondence:*** olesiagrechko@mail.ru. O. Yu. Grechko

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or sodium currents with stable parameters taken as initial (control) parameters were recorded in 3-5 min after solution replacement. Then, bathing solution was replaced with a solution containing the test substance. When changes in ionic currents induced by the test substances stabilized (in 2-3 min), measurements were repeated. Then, the solution was replaced with the initial one and the dynamics of current recovery (washout) was observed.

Current-voltage curves (CVC) of membrane ionic channels and concentration–effect curves were constructed from these data using a computer. The initial current parameters were taken as 100% and changes induced by the test compound were expressed in percents of initial values and processed statistically using Student's *t* test.

RESULTS

Ru-1203 in a concentration of 500 μM induced a significant decrease in sodium current amplitude (to 51.4±4.8% from the control; Table 1; Fig. 1, *a*, upper curve). Interestingly, norBNI in a concentration of 5 μM inhibited inward sodium current, but did not change significantly the effects of RU-1203 (Table 1; Fig. 1, *b*).

Evaluation of the effects of norBNI on functional activity of voltage-gated calcium channels revealed insignificant decrease in their amplitude and acceleration of inactivation kinetics (Fig. 1, *c, 3*). RU-1203 (500 μM) significantly suppressed calcium channels of the neuronal membrane and this effect was not abolished by preincubation with norBNI (Table 1; Fig. 1, *d*).

Figure 1 (*f*) shows modulation of calcium currents under the effect of RU-1203 (500 μM). norBNI-mediated changes in potassium currents were appreciable, but less pronounced (Fig. 1, *e, 1* and *f, 3*). It should be noted that norBNI insignificantly increased and in some experiments reduced the amplitude of slow potassium currents. The kinetics of the current remained unchanged under these conditions, but unspecific leakage currents decreased. Similarly to sodium and calcium currents, the inhibitory effects of RU-1203 were not abolished by RU-1203.

It can be concluded that RU-1203 is similar to local anesthetics and antiarrhythmic preparations by the channel-blocking effects [1]. The molecular mechanism of ionic channel suppression by the test compound in this experimental model is evidently related to direct blockade of ionic channels. The current-inhibitory effects can be determined by reducing the number of active channels due to binding of the compound molecule with channels structure, most likely with S_5-S_6 segments in the channel mouth [9] and shortening of open state time or decrease in opening frequency (in case of modulation of current kinetics). It is known many membrane-active compounds acts not only after penetration into open ionic channel on the cell membrane, but also within the lipid bilayer by acting on S_4-segment of α-subunit of voltage-operated channels, a strain sensor responsible for conformation rearrangements and ionic channel gate opening [12].

Whole-cell patch-clamp experiments on sensory neurons of rat rectum showed that synthetic κ-opioid receptor ligands U50,488, spiradoline, *etc*. in micromolar concentrations effectively block ionic channels without involving κ-opioid receptor structures [11,15].

Opioid analgesics morphine, tramadol, promedol, and butorfanol apart from receptor-mediated effects can also produce a direct nonspecific influence on potential-dependent ionic channels of the neuronal membrane [2,3,5].

An important role in transmission of nociceptive information is played by functional properties of potential-dependent sodium, calcium, and potassium ionic channels and electrical synapses synchronizing activity of voltage-operated channels thus coordinating activity of local neuronal groups. They transmit the signal from cell to cell practically without delay due to narrow synaptic gap and membrane pores consisting of connexin protein. Electrical and chemical synapses are located in immediate proximity from each other and probably ensure electrotonic and chemical

TABLE 1. Individual and Combined (Preincubation with norBNI) Effects of RU-1203 and norBNI on Ionic Channels of *Lymnaea stagnalis* Neurons (%; *M*±*tm*)

Currents, statistical parameters	RU-1203, 500 μM	norBNI, 5 μM	norBNI, 5 μM+RU-1203, 500 μM
INa	51.4±4.8	89.7±4.4	48.5±5.6
ICa	58.8±5.2	91.8±3.8	54.3±4.9
IKs	62.1±6.3	85.7±5.5	53.1±4.6
IKf	64.6±5.3	89.9±4.3	60.6±7.0

Note. INa, ICa, IKs, and IKf are amplitudes of sodium, calcium, and slow and fast potassium currents, respectively; *M* is mean current amplitude (% of initial) under the effect of the test substances, *tm* is confidence interval (*p*=95%), *n*=5-11.

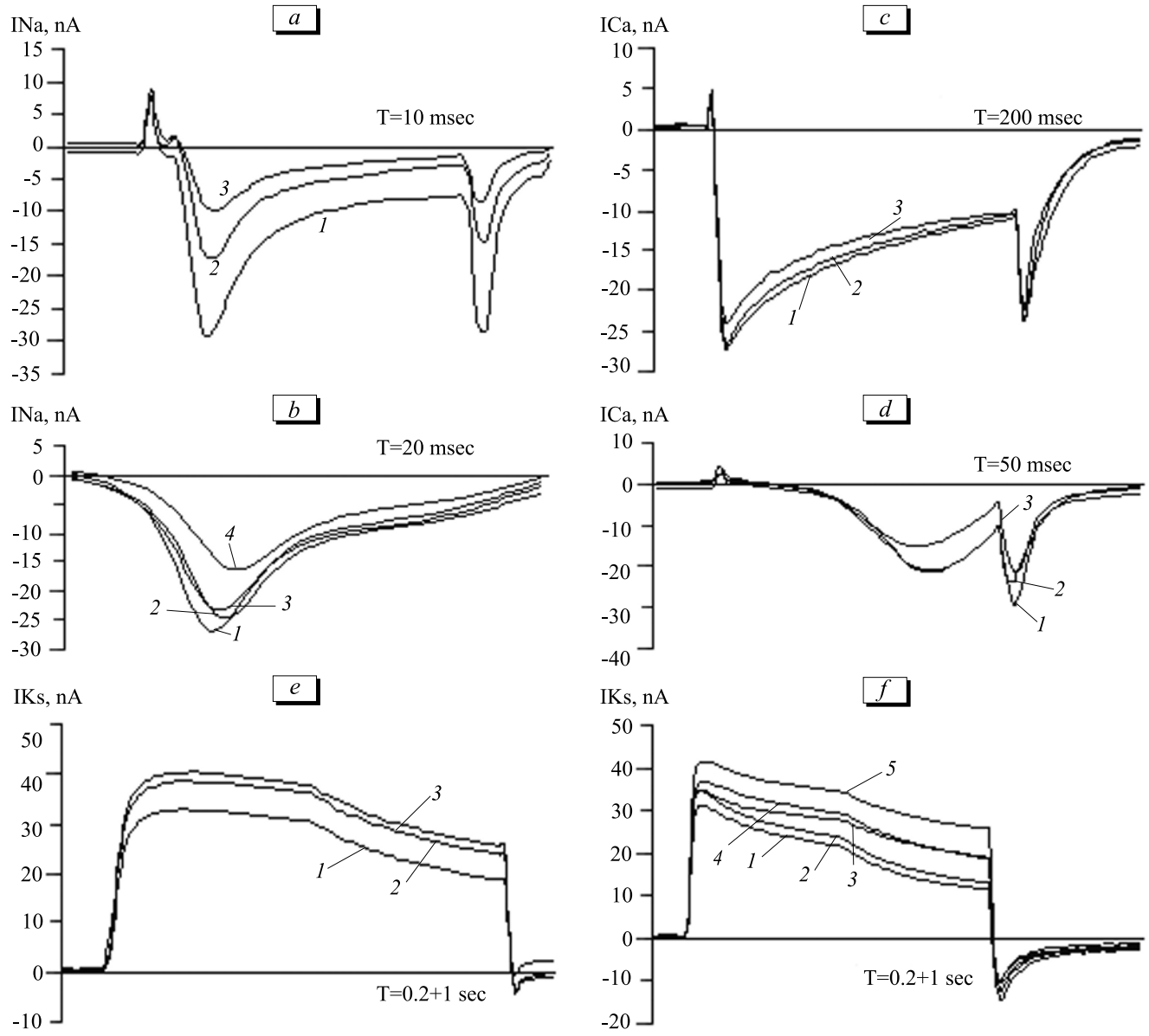

Fig. 1. Changes of sodium, calcium, and potassium currents in *Lymnaea stagnalis* neurons induced by RU-1203, norBNI, and RU-1203 after preincubation with norBNI. *a*: changes in neuronal INa under the effect of RU-1203 in concentration of 500 μM: *1*) control, *2*) washout, *3*) 500 μM. *b*: changes in BAX Na-channels in neuronal membrane under the effect of 5 μM norBNI and 500 μM RU-1203 after preincubation with norBNI: *1*) control, *2*) norBNI; *3*) washout, *4*) norBNI+RU-1203. *c*: changes in neuronal ICa under the effect of 5 μM norBNI: *1*) control, *2*) washout, *3*) norBNI. *d*: changes in BAX Ca-channels in neuronal membrane under the effect of 500 μM RU-1203 after preincubation with 5 μM norBNI: *1*) control, *2*) washout, *3*) norBNI+RU-1203. Abscissa: time corresponding to saw-shape amplitude shift from -40 to 10 mV. *e*: changes in neuronal IKs under the effect of 5 μM norBNI: *1*) norBNI, *2*) control, *3*) washout. *f*: changes in neuronal IKs under the effect of 5 μM norBNI, 500 μM RU-1203, and after preincubation with norBNI: *1*) norBNI+RU-1203, *2*) RU-1203, *3*) washout, *4*) norBNI; *5*) control.

regulation of synchronization of neuronal activity. It is hypothesized that electrical contacts realize local (within 200 μ) synchronization, while chemical contacts are involved later and ensure distant synchronization of impulse activity [8]. The direct modulation of potential-dependent ionic channels by a new derivative of condensed benzimidazole systems RU-1203 can considerably contribute to the realization of its neurotropic effects.

At the same time, the interaction with κ-receptors and transmission of a modulating signal to ionic channels through G-proteins is believed to be the main mechanism of membranotropic effect of κ-selective ligands [11,13,14].

There are data that typical κ_1-, κ_2-, and κ_3-opioid receptor agonists U50,488, bremazocine, and naloxone benzoylhydrazone and partial μ-opioid receptor agonist/agonist and κ-opioid receptor agonist butorphanol

can modulate functional activity of sodium, calcium, and potassium voltage-operated ionic channels in different cell types, from hippocampal neurons to neurons localized in spinal root ganglia. This effect of κ-receptor ligands is abolished by preincubation with naloxone, κ-selective antagonist norBNI, and also by pretreatment with pertussis-toxin [1,9].

Our findings did not disprove the receptor-mediated mechanism of the inhibitory effects of RU-1203 on neuronal membrane ionic channels. The fact that norBNI did not abolish membranotropic effects of the compound can also be explained by the choice of the experimental model. The existence of the κ-opioid system in mollusks is now convincingly proven, but the expression of κ-receptors and other opioid receptors in *Lymnaea stagnalis* neurons was not demonstrated. Previous studies showed that morphine, promedol, butorphanol, buprenorphine, U50,488, and tramadol produced a similar inhibitory effects on ionic channels of *Lymnaea stagnalis* neurons and the effects of these substrances were not abolished by injection of naloxon, *i.e.* were naloxon-irreversible [2,3,5].

Insignificant and reversible suppression of sodium, calcium, and in most cases potassium ionic channels under the effect of κ-opioid antagonist norBNI in a concentration range of 0.1-10.0 μM was similar to the effect of RU-1203 in concentrations 10-20 μM and higher and naloxone [3]. These findings probably attest to a direct influence of norBNI on functions of ionic channels in *Lymnaea stagnalis* neurons and antagonistic activity of κ-selective agonist in this experimental model. A similar assumption was made for explaining the influence of naloxon on ionic channels in mollusk neurons [1]. According to published data, naloxon under certain conditions can act as an opioid receptor agonist [4]; this activity of norBNI was never reported, but cannot be excluded.

Thus, our findings drove us to the following conclusions:

- RU-1203 in a concentration of 500 μM and κ-opioid receptor antagonist norBNI in concentrations of 0.1-10.0 μM produced a pronounced membranotropic effects on *Lymnaea stagnalis* neurons; manifested in inhibition of ionic channels of voltage-operated ionic channels, which probably constitute the basis of their neurotropic effects.
- The amplitude of ionic currents almost completely restored within 5-7 min, which attested to medium binding strength between their molecules and molecular structures of the membranes or channels.
- Recording of all ionic currents showed that RU-1203 and norBNI reduced nonspecific leakage currents, which can be interpreted as membrane-stabilizing effect.
- κ-Opioid receptor antagonist norBNI in concentrations 0.1-10.0 μM insignificantly suppressed the amplitude of all ionic currents, but did not abolish the current-inhibiting effects of RU-1203.

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