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Molecular Mechanism of Modulation of Nociceptive Neuron Membrane Excitability by a Tripeptide

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Abstract—Using the whole-cell patch-clamp method, the ability of arginine-containing tripeptide Ac-RER-NH_2, dipeptide Ac-RR-NH_2, and free Arg molecule to modulate the membrane excitability of nociceptors was studied. Extracellular Ac-RER-NH_2 upon interaction with the outer membrane of the nociceptive neuron decreases the Z_{eff} value of the activation gating system of $Na_v1.8$ channels. Thus, the tripeptide Ac-RER-NH_2 can be considered as a new effective and safe analgesic.

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The discovery of slow sodium channels has allowed a new approach to studying the mechanisms of nociception [1, 2]. These channels can be coupled to membrane receptors, thus forming a specific receptive field on the neuron membrane, an example of which is the targeted modulation of NaV1.8 channels, which are responsible for coding the pain signal in the nociceptive neurons, serotonin receptors [3], and opioid-like receptors [4]. Activation of opioid-like receptors by comenic acid, a gamma-pyrone derivative, makes it possible to selectively disable only the high-frequency component of the pulse activity, specifically coding the nociceptive signal of the sensory neuron membrane, without causing adverse side effects [5].

Compounds of peptide and nonpeptide nature can be promising pharmacological substances activating this mechanism of membrane signaling. We proposed comenic acid, a gamma-pyrone derivative, as an active substance of the new Russian non-opioid analgesic Anoceptin [5]. The latter has successfully passed the first phase of clinical trials, which allowed us to accumulate a unique experience in developing completely new and effective analgesics.

Another direction of our studies involves the development of peptide analgesics based on rabbit defensin molecules. As has been shown previously [6–9], the extracellular interaction of endogenous antibiotics defensin NP-1 (K_d = 2 nM) and defensin NP-4 (K_d = 80 pM) with the outer membrane of rat dorsal root ganglion neurons leads to a decrease in the effective charge of the activation gating system of slow sodium channels $Na_v1.8$ (Z_{eff}), which indicates the ability of these molecules to modulate the voltage sensitivity of these channels. Since the molecules of the studied rabbit defensins are composed of 33 amino acid residues and have a complex structure, we have attempted to isolate the fragments of these molecules in the form of short peptides that could be fairly easily synthesized but, at the same time, retained the necessary range of physiologically important properties (primarily the analgesic effect). It was found that the synthetic hexapeptides Ac-PRERRA-NH_2, Ac-PRARRA-NH_2, and Ac-PKEKKA-NH_2 (PRERRA sequence is a fragment of the native form of the defensin NP-1 molecule) at endogenous concentrations (100 nM) decreased the voltage sensitivity of $Na_v1.8$ channels [10].

Since the substitution of Glu with Ala (the transition from Ac-PRERRA-NH_2 to Ac-PRARRA-NH_2) had little effect on the modulating properties of the peptide [10], it can be concluded that the presence in the attacking molecule of the amino acid residue Glu is not a prerequisite that determines the ability of the peptide to bind to the channel, which disagrees with our earlier standpoints [7]. On the other hand, the retention of the observed effect upon the transition from Ac-PRERRA-NH_2 to Ac-PKEKKA-NH_2 indicates that a positively charged functional group is involved in the formation of a ligand-receptor complex. Amino acid residues Arg and Lys carry a positive charge in the side chain at physiologically appropriate conditions, and the reliably established ability of the Ac-PKEKKA-NH_2 molecule to reduce Z_{eff} is suggestive of potential interchangeability of Arg and Lys in the binding of hexapeptides by the receptor modulation mechanism [11] to their molecular target that is a fragment of the amino acid sequence of $Na_v1.8$ channel [10].

Apparently, the amino acid residue Arg plays the key role in the mechanism of ligand–receptor binding of the Ac-PRERRA-NH_2 molecule. An important task is to find the shortest peptide containing the spec-

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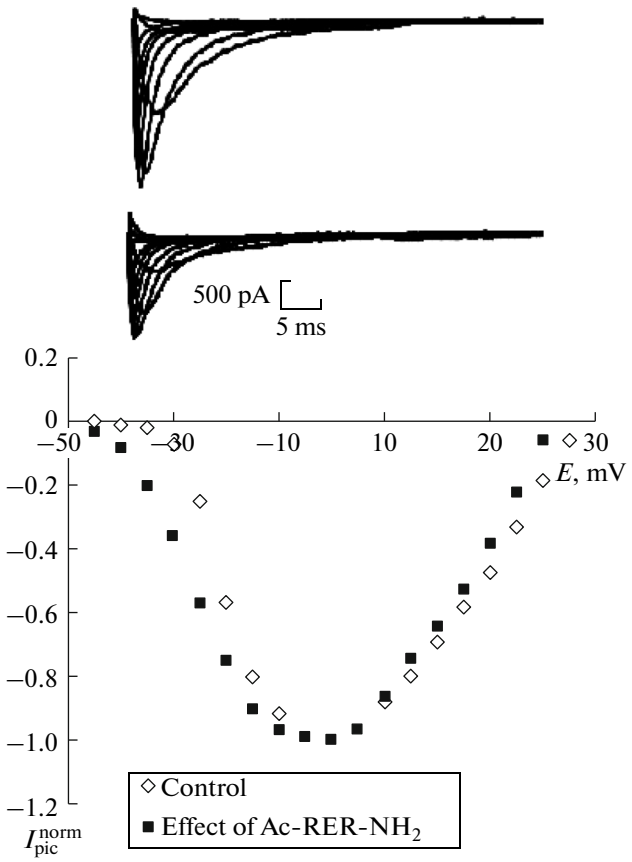

Fig. 1. Normalized values of peak current–voltage characteristics of sodium channels $Na_v1.8$ built on the basis of the control experimental data and after the addition of 1 μM tripeptide Ac-RER-NH_2 to the intracellular solution. Top right—the curves of slow $Na_V1.8$ currents obtained in the control experiments and in the presence of 1 μM tripeptide Ac-RER-NH_2. The test voltage range varied from −60 to 45 mV, step 10 mV. In all records the maintained voltage with a duration of 500 ms was 110 mV. The leakage and capacitive currents were subtracted using the software.

ified amino acid residue and capable of reducing the voltage sensitivity of $Na_v1.8$ channels. Such a peptide may apparently exhibit the same analgesic properties as comenic acid but, due to its endogenous nature, may be safer and more efficient. For its development, it was necessary to investigate in detail the mechanisms of action of arginine-containing peptides on the nociceptive neuron membrane. This was the subject of the current study.

Using the whole-cell patch-clamp technique, we studied the ability of Ac-RER-NH_2, Ac-RR-NH_2, and free Arg molecules to modulate the nociceptor membrane excitability, i.e., the ability to reduce the voltage sensitivity of slow sodium channels $Na_v1.8$, which are responsible for coding pain signals.

Experiments were performed on cultured sensory neurons isolated from the regions of L_5–S_1 spinal ganglia of newborn Wistar rats using the standard solutions [12]. Culturing isolated neurons for 2 h in standard culture media in a 95% CO_2 atmosphere allows obtaining intact cells [12, 13] whose membrane retains the physiological nociceptive characteristics. The peptides were obtained by the classical peptide synthesis, which was performed using the reagents and amino acid derivatives from Sigma Chemical Co. (United States) and Iris Biotech GmbH (Germany) and solvents from Ekos-1 and Cryochrom (Russia). The final product was characterized by analytical HPLC (purity >95%) and mass spectrometry. The results were statistically processed using Student's t test. Differences at $p \le 0.05$ were regarded significant.

The action of the Ac-RER-NH_2 molecule at a concentration of 1 μM on the outer surface of the neuronal plasma membrane changed the effective charge (Z_{eff}) of the activation gating system of $Na_v1.8$ channels (Figs. 1, 2). This parameter is an index reflecting the effect of the studied agents. A decrease in Z_{eff} leads to a reduction in the repetitive firing frequency of the nociceptive neuron membrane, which, in turn, leads to the blockade of pain signals [5, 12]. The Z_{eff} value was quantified by the conventional procedure [12]. When a sequence of voltage steps (E) was applied to the membrane, amplitude (peak) current values (I_{max}) were recorded, which can be expressed as a function of I_{max} (E). In Fig. 1, this dependence is presented as a normalized function. Then, the use of Almers method [5, 12] makes it possible to estimate the effective charge of the activation gating system of slow sodium channels with a sufficient accuracy (Fig. 2).

The main result of our study was the determination of the minimum effective length of the amino acid sequence of the arginine-containing peptides. We have found that the Z_{eff} value decreased under the influence of the tripeptide Ac-RER-NH_2, whereas the dipeptide Ac-RR-NH_2 and the free Arg molecule had no such an effect (table).

The full optimization of the geometric parameters of isolated molecules of the tripeptide Ac-RER-NH_2 and the dipeptide Ac-RR-NH_2 was performed by the semi-empirical PM3 method and the restricted Hartree–Fock method *ab initio* with the 6-31G* basis [14] using the GAMESS software [15]. Quantum chemical calculations have shown that the tripeptide molecule was stabilized by the salt bridge between the guanidine group in the side chain of Arg1 and the carboxyl group of the side chain of Glu2 and three intramolecular hydrogen bonds: the hydrogen bond between the nitrogen atom of the guanidine group of Arg3 and the carbonyl oxygen atom of the amidated C-terminus of the peptide molecule. These interactions significantly restrict the conformational mobility of the molecule.

The main contribution to the binding energy of the tripeptide Ac-RER-NH_2 to the channel is probably made by the formation of the intermolecular ion-ionic bond with the involvement of the guanidine group of the Arg3 residue and the nucleophilic site in the modulated receptor. This assumption is confirmed by the steric availability of the guanidine fragment of Arg3 as

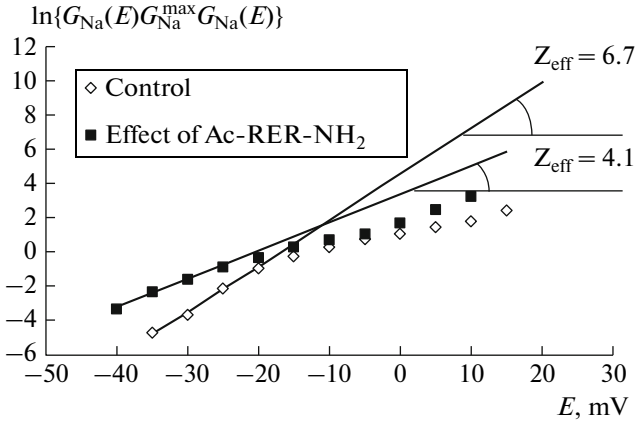

Fig. 2. Effect of the tripeptide Ac-RER-NH_2 on the effective charge of the activation gating system of slow sodium channels. The exponential function represented on a logarithmic scale (ordinate axis) allows the Z_{eff} value to be determined by the slope of the asymptote to the initial sections of these functions in the control experiment and after adding 1 μM tripeptide Ac-RER-NH_2 to the intracellular solution.

well as by the fact that the ionized functional groups of the side chains of Arg1 and Glu2 form a salt bond with each other, which must be broken to allow these groups to effectively interact with the receptor. The function of the amino acid residue of Glu2 apparently consists in stabilizing the active conformation of the tripeptide molecule due to the formation of the intramolecular ionic bond.

The Ac-RR-NH_2 dipeptide molecule also contains two sterically available positively charged guanidine groups; however, this molecule, as well as the free Arg molecule, is unable to reduce the Z_{eff} value.

The most likely explanation for the absence of the observed effect in Arg and Ac-RR-NH_2 molecules is that the energy of formation of the intermolecular ion-ionic ligand–receptor bond alone is insufficient for the activation of the ligand binding to the receptor, which leads to a decrease in Z_{eff}. Van der Waals interactions and the formation of intermolecular hydrogen bonds can also contribute to the total energy of the ligand–receptor complex formation. The Ac-RR-NH_2 molecule is elongated due to electrostatic repulsion of guanidine fragments carrying charges of the same sign, whereas the Ac-RER-NH_2 molecule is more compact due to the presence of stabilizing intermolecular interactions and has an ellipsoid shape, which makes this molecule more complementary to its binding site.

Quantitative assessment of the effect of the test substances on the effective charge of slow sodium channels

Substance	Concentration, μM	Z_{eff}, ($\bar{X} \pm SD$)	Number of experiments
Control		6.6 ± 0.3	24
Ac-RER-NH_2	1	4.6 ± 0.4*	14
Ac-RR-NH_2	1	6.2 ± 0.4	14
Arg	1	6.3 ± 0.3	12

* $p < 0.05$ compared to the control.

Thus, the shortest peptide that can exhibit potential analgesic properties is the tripeptide Ac-RER-NH_2. However, its effective concentration is 10 times higher than that of the hexapeptide Ac-PRERRA-NH_2, comprising the RER sequence, and 6 orders of magnitude higher than that of the NP-1 molecule.

The results of this study indicate that the interaction of the Ac-RER-NH_2 molecule with the nociceptive neuron membrane has the same effect (reduction of Z_{eff} value of the activation gating system of $Na_v1.8$ channels) as comenic acid, which is an active substance of the highly effective and safe non-opioid analgesic of non-peptide nature Anoceptin, developed by us. Another common feature of Ac-RER-NH_2 and Anoceptin is that they have an electrophilic site that is involved in the formation of the intermolecular ion-ionic bond, which makes the major contribution to the energy of the ligand–receptor complex formation. In the Ac-RER-NH_2 molecule, this is the guanidine group of the side chain of Arg, and in comenic acid molecule this is the chelated Ca^{2+} atom, because this molecule binds to the receptor in the form of a salt of a chelate complex with Ca^{2+} [5]. Thus, the Ac-RER-NH_2 molecule can be considered as a possible new endogenous analgesic.

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