Pathophysiology 26 (2019) 245–252

Contents lists available at ScienceDirect

Pathophysiology

journal homepage: www.elsevier.com/locate/pathophys

Molecular mechanisms and signaling by comenic acid in nociceptive neurons influence the pathophysiology of neuropathic pain

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ARTICLE INFO

Article history:
Received 14 March 2019
Received in revised form 10 June 2019
Accepted 13 June 2019

Keywords:
Comenic acid
Nociceptive neuron
$Na_V1.8$ channels
Signaling cascade

ABSTRACT

Comenic acid (CA), a specific agonist of opioid-like receptors, effectively and safely relieves neuropathic pain by decreasing the $Na_V1.8$ channel voltage sensitivity in the primary sensory neuron membrane. CA triggers downstream signaling cascades, in which the Na,K-ATPase/Src complex plays a key role. After leaving the complex, the signal diverges 'tangentially' and 'radially'. It is directed 'tangentially' along the neuron membrane to $Na_V1.8$ channels, decreasing the effective charge of their activation gating system. In the radial direction moving towards the cell genome, the signal activates the downstream signaling pathway involving PKC and ERK1/2. A remarkable feature of CA is its ability to modulate $Na_V1.8$ channels, which relieves neuropathic pain while simultaneously stimulating neurite growth via the receptor-coupled activation of the ERK1/2-dependent signaling pathway.

1. Introduction

Hundreds of millions people the world over suffer from chronic pain, and the number is steadily increasing. One of the most important issues that needs to be addressed is unravelling how 'pain as a symptom,' a crucial protective function for any organism, transforms into pathological 'pain as a disorder.' Identification of the specific effect of comenic acid (CA) on the voltage sensitivity of the slow sodium $Na_V1.8$ channels responsible for nociceptive signal coding has made it possible to apply CA in development of novel approaches for neuropathic pain relief [1].

Comenic acid (Fig. 1) (CA) is a specific agonist of opioid-like receptors in the sensory neuron membrane. The signal triggered by activation of these receptors is transduced to $Na_V1.8$ channels, which 'encode' the responses of nociceptors [1,2]. Over the last twenty years, we have significantly improved our understanding of the physiological role of this novel membrane signaling pathway, which comprises opioid-like receptors coupled to slow sodium $Na_V1.8$ channels via the Na,K-ATPase/Src complex as the signal transducer. The pathway is distinct from and additional to the known mechanism of the opioidergic system functioning that involves G proteins. Activation of the opioid-like receptor by CA, which triggers the signaling pathway directed at the $Na_V1.8$ channels, is responsible for the effectiveness and safety of our novel analgesic Anoceptin®, which is potent enough to relieve the kind of severe neuropathic pain otherwise relieved exclusively by opioids.

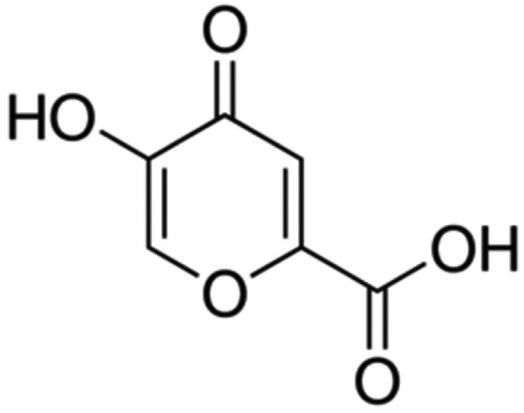

Fig. 1. Structural formula of comenic acid.

It should be stressed once again that the role of the signal transducer in this pathway is played by the Na,K-ATPase/Src complex [3–6]. The receptor-mediated signal triggered by CA is also directed

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https://doi.org/10.1016/j.pathophys.2019.06.003
0928-4680/

to the sensory neuron genome, which regulates neurite growth [5]. Little research has been done to investigate the other units of this intracellular cascade process.

It has been demonstrated that ouabain at very low (nanomolar) concentrations activates the non-pumping Na,K-ATPase function in various cell types [7–10]. These concentrations correspond to the endogenous level of ouabain detected in human blood [11], which seems to be sufficiently high to trigger the intracellular cascades that use the Na,K-ATPase/Src complex as the signal transducer instead of G proteins. This complex performs cell-specific signal transduction, activating the Raf/MEK/ERK, PLC/PKC, and PI3K/Akt pathways, as well as Ca^{2+} signaling and ROS generation [12–14]. It should be noted that ouabain-triggered signal transduction in cells capable of proliferative growth may activate either Src-dependent or Src-independent pathways [8,15–17]. Src-independent ouabain-induced activation of the ERK and Akt pathways with the participation of PI3K and PKC has been demonstrated in LM-α3-1 cells [17].

The Src-dependent p38 MAPK pathway was found to be activated in sensory neurons by endogenous ouabain concentrations via a transducer-coupled mechanism [18]. However, the Src-dependent receptor-coupled intracellular signaling triggered by CA upon its binding to the opioid-like receptor remains uninvestigated. The objective of this study is to elucidate the role of a number of proteins in this receptor-coupled downstream signaling cascade in primary sensory neurons.

2. Methods

2.1. Organotypic nerve tissue culture method

Experiments were conducted on 10- to 12-day-old chick embryo dorsal root ganglia (DRG) explants cultured in collagen-coated 40-mm Petri dishes in a humidified CO_2 incubator (Sanyo, Japan) for 3 days at 36.5 °C and 5% CO_2. The culture medium was prepared from 45% Hank's solution, 40% Eagle's medium, and 10% fetal bovine serum supplemented with glucose (0.6%), glutamine (2 mM), and gentamicin (100 units/mL) [5,19]. Comenic acid (10 nM) was used to trigger the signaling mechanism, the initial unit of which is the opioid-like membrane receptor. Subsequent units of this downstream process were detected using the following specific inhibitors added to the culture medium 30 min prior to CA: protein kinase A (PKA) inhibitor KT5720 (1 μM); protein kinase C (PKC) inhibitor tamoxifen (10 μM); p38 mitogen-activated protein kinase (p38 MAPK) inhibitor SB239063 (1 μM); extracellular signal-regulated kinases 1/2 (ERK1/2) inhibitor PD98059 (1 μM). All inhibitors were purchased from Sigma (USA).

Explants cultured in the culture medium only were used as controls. An Axio Observer Z1 microscope (Carl Zeiss, Germany) was used to visualize the objects. The images obtained were analyzed using ImageJ and ZEN_2012 software. Neurite growth was evaluated using the morphometric method. The area index (AI) was calculated as the ratio of the explant growth area compared to the area of the central zone of a ganglion [20]. The AI of control explants was designated as 100%. Experiments were carried out using the equipment of the Confocal Microscopy Collective Use Center (Pavlov Institute of Physiology of the Russian Academy of Sciences).

2.2. Patch-clamp method

Experiments were designed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). The Local Committee for Animal Care and Use at Pavlov Institute of Physiology of the Russian Academy of Sciences approved all experimental procedures with the animals, permit number №10/03 (20.04.2017). Animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press). All animals were obtained from the Biocollection of Pavlov Institute of Physiology.

Electrophysiological experiments were performed using the patch-clamp method in the 'whole-cell recording' configuration [21] on dissociated sensory neurons obtained using a short-term cell culture technique [22]. The neurons were isolated from the L_5-S_1 region of the spinal cords of newborn Wistar rats. Single intact nerve cells were obtained by culturing the neurons in standard culturing medium for 2 h in a humidified CO_2 incubator. The modified dissociated sensory neuron culturing technique was described in more detail earlier [15,19].

Slow sodium $Na_V1.8$ currents were investigated using the following solutions (concentrations in mM). Extracellular solution (pH 7.4): Choline Cl, 70; NaCl, 65; HEPES Na, 10; $CaCl_2$, 2; $MgCl_2$, 2; TTX, 0.0003. Intracellular solution (pH 7.2): CsF, 100; CsCl, 40; HEPES Na, 10; NaCl, 10; $MgCl_2$, 2. All potassium currents were thus excluded due to the absence of potassium ions, while intracellular fluoride ions blocked the calcium currents [22,23]. Extracellular tetrodotoxin eliminated all fast tetrodotoxin-sensitive sodium channels, which made it possible to register the responses of slow tetrodotoxin-resistant $Na_V1.8$ sodium channels only. All reagents were purchased from Sigma (USA).

Changes in the voltage sensitivity of $Na_V1.8$ channels were estimated based on evaluation of the effective charge (Z_{eff}) of a $Na_V1.8$ channel activation gating device using the Almers's method [24]. This approach was described in detail earlier [1,2,19].

2.3. Statistical analysis

The data were analyzed with STATISTICA 10.0 (StatSoft, USA) using the Student's *t*-test and expressed as the mean value ± SEM. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Organotypic nerve tissue culture method

Comenic acid (CA) exhibits a pronounced neurite-stimulating effect due to its interaction with the opioid-like receptor coupled to Na,K-ATPase, which functions as the transducer of signals directed at the $Na_V1.8$ channels and the cell genome. CA at a concentration of 10 nM stimulated outgrowth of DRG neurons by 56 ± 5% ($n = 32$, $p < 0.05$) (Fig. 2).

To investigate the downstream signaling pathways underlying the neurite-stimulating effect of CA, a number of inhibitors were applied to the DRG cultures. The concentrations of PKA, PKC, p38 MAPK, and ERK1/2 inhibitors were chosen such that the inhibitors applied alone (without CA) had no effect on neurite growth (Fig. 3). Application of the inhibitors made it possible to detect the units of the receptor-mediated downstream signaling cascade triggered by CA.

3.2. Involvement of PKC and PKA in the neurite-stimulating effect of CA

Explants were preincubated with KT5720 (a selective PKA inhibitor, 1 μM, 30 min) before treatment with CA (10 nM). KT5720 did not block the neurite growth induced by CA (Fig. 4). The AI of the experimental explants corresponded to that obtained with application of CA alone and was 56 ± 5% ($n = 32$, $p < 0.05$) above control, which demonstrates that PKA does not participate in the downstream signaling pathway triggered by CA.

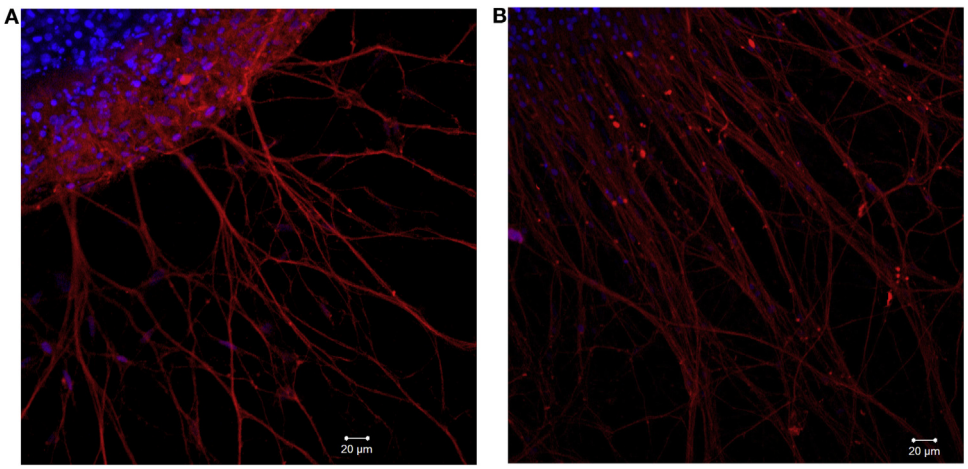

Fig. 2. Stimulating effect of comenic acid on DRG neurite growth. Fragments of DRG explant growth zone (third day of culturing). DRG explants treated with comenic acid were fixed and immunostained using an anti-neurofilament antibody (red). Nuclei were counterstained using DAPI (blue). Scale bar 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
A – control.
B – comenic acid (10 nM).

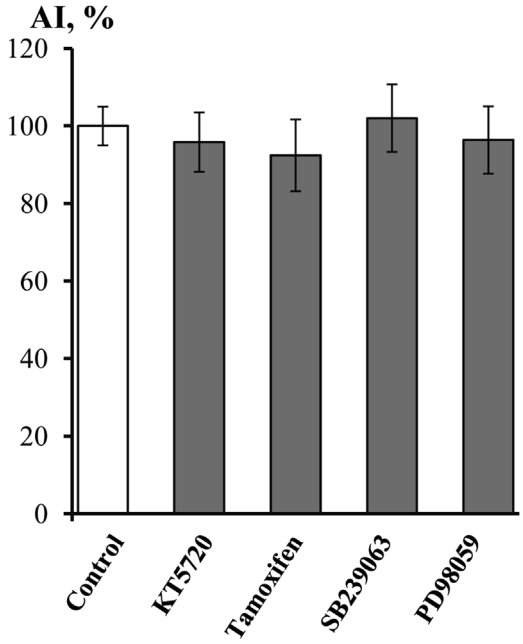

Fig. 3. Effect of various inhibitors on DRG neurite growth. Explants were cultured in medium containing KT5720 (1 μM), tamoxifen (10 μM), SB239063 (1 μM), or PD98059 (1 μM).
Ordinate axis: area index of explants (AI, %).

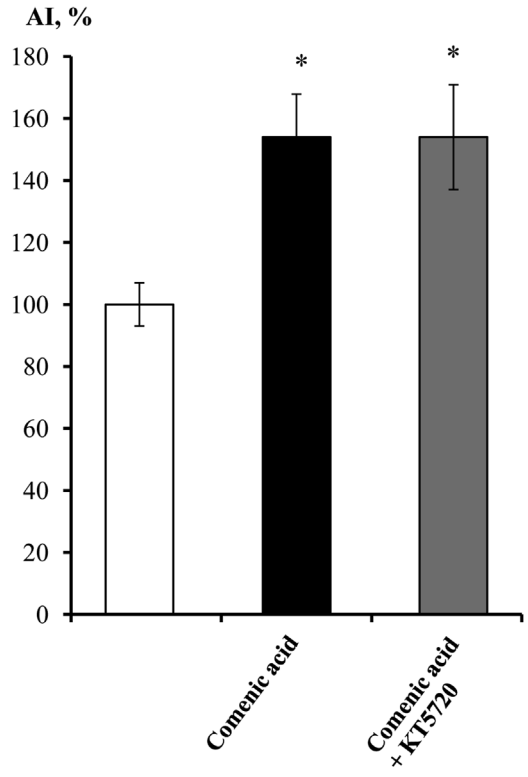

Fig. 4. Change of area index of DRG explants upon action of comenic acid (10 nM) in presence of the PKA inhibitor KT5720 (1 μM).
Ordinate axis: area index of explants (AI, %).
Significant differences ($p < 0.05$) are indicated with asterisks.

Tamoxifen (a selective PKC inhibitor, 10 μM, 30 min) significantly diminished the CA-induced neurite growth of DRG neurons (Fig. 5). The AI of experimental explants did not differ from those of the controls. The result obtained unambiguously indicates that PKC is involved as a consecutive unit in the receptor-mediated cascade process triggered by CA.

These data suggest that the neurite-stimulating effect of CA does not depend on PKA but is PKC dependent.

3.3. Receptor-mediated activation of p38 MAPK and ERK1/2 signaling pathways by CA

Explants were treated with SB239063 (a selective p38 MAPK inhibitor, 1 μM, 30 min) before application of CA (10 nM). As shown in Fig. 5, SB239063 did not block the neurite-stimulating effect of CA. The AI was 56 ± 5% ($n = 32$, $p < 0.05$) above control, similar to the values obtained upon treatment with CA alone (Fig. 6), which indicates that the stimulating effect of CA is not due to activation of the p38 MAP kinase signaling pathway.

The role of ERK1/2 in neurite growth regulation by CA was also investigated. Explants were preincubated with PD98059 (a selective ERK1/2 inhibitor, 1 μM, 30 min) before treatment with CA (10 nM). The neurite-stimulating effect of CA was blocked by

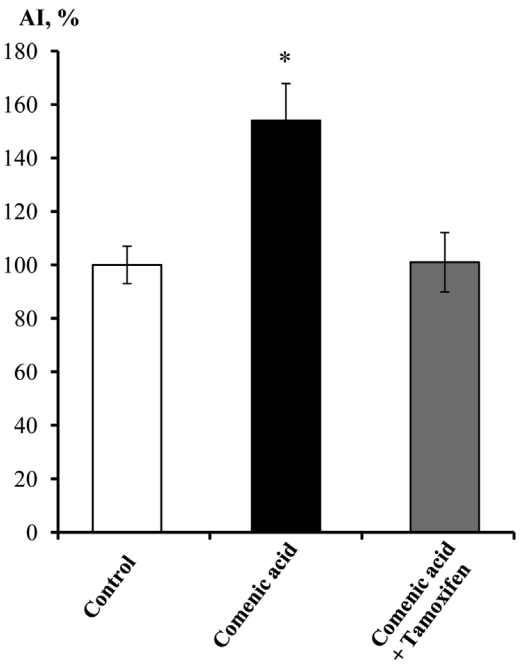

Fig. 5. PKC-dependent activation of DRG neurite growth triggered by comenic acid. Tamoxifen (10 μM) blocked the neurite-stimulating effect of comenic acid (10 nM).
Ordinate axis: area index of explants (AI, %).
Significant differences ($p < 0.05$) are indicated with asterisks.

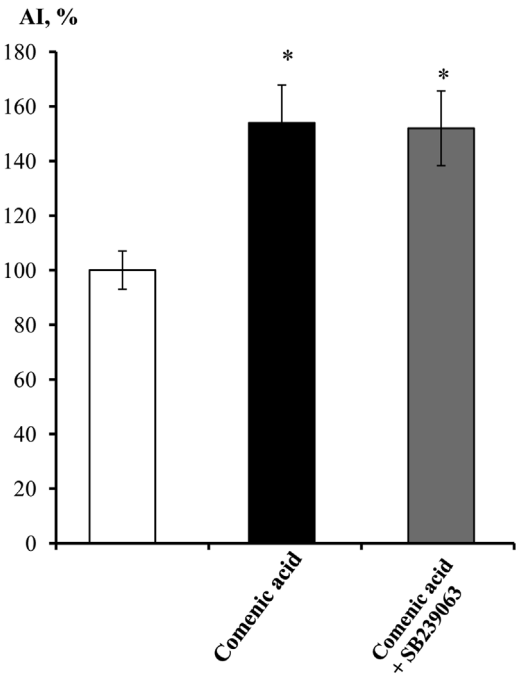

Fig. 6. Combined action of comenic acid (10 nM) and p38 MAPK inhibitor SB239063 (1 μM).
Ordinate axis: area index of explants (AI, %).
Significant differences ($p < 0.05$) are indicated with asterisks.

PD98059 (Fig. 7). The AI of the experimental explants was close to that of the controls.

Thus, the growth-promoting effect of CA is mediated by ERK1/2. It can be concluded that binding of CA to the opioid-like receptor activates the Na,K-ATPase-Src-PKC-ERK1/2 pathway.

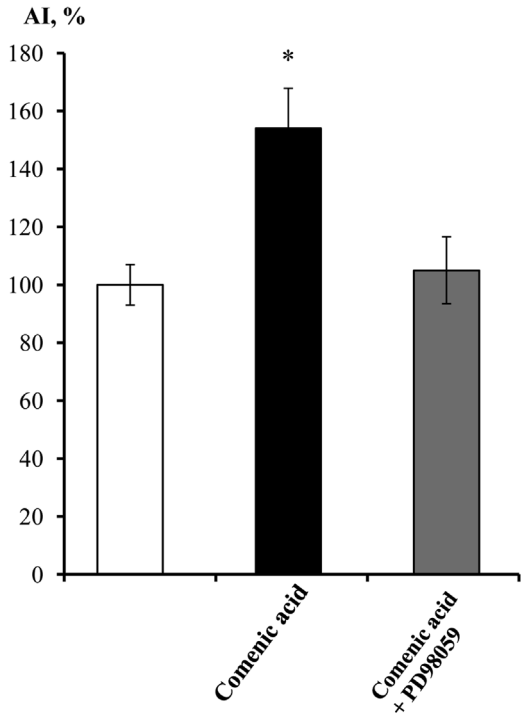

Fig. 7. Participation of ERK1/2 in the neurite-stimulating action of comenic acid. Blocking ERK1/2 with PD98059 (1 μM) eliminated the neurite growth triggered by comenic acid (10 nM).
Ordinate axis: area index of explants (AI, %).
Significant differences ($p < 0.05$) are indicated with asterisks.

3.4. Investigation of involvement of PKC in the modulation of $Na_V1.8$ channels by CA

The patch-clamp method was used to investigate the involvement of PKC in tangential signal transduction along the neuron membrane from the opioid-like receptor to the $Na_V1.8$ channels. The families of $Na_V1.8$ sodium currents recorded in control experiments and after extracellular application of 100 nM CA are shown in Fig. 8A. The normalized peak current-voltage characteristics of sodium currents plotted using the regular protocol [25] in a control experiment and after application of CA are displayed in Fig. 8B.

Another set of experiments was performed with the intracellular solution containing tamoxifen, a specific PKC inhibitor, at 10 μM. Tamoxifen was present in the intracellular solution during all of the tests, including the controls. The tamoxifen concentration was chosen based on the results presented above, as it was demonstrated in organotypic tissue culture that at a concentration of 10 μM, tamoxifen blocked the receptor-mediated effect of CA on neurite growth. The families of $Na_V1.8$ sodium currents recorded before and after application of 100 nM CA are shown in Fig. 8C, while their normalized current-voltage functions are displayed in Fig. 8D.

Application of CA results in a shift of the current-voltage functions to the right along the voltage axis in both sets of experiments (Fig. 8B, D), which indicates that the agent interacts with the opioid-like receptor and activates the receptor-mediated signaling mechanism [1,2,5]. The effective charge of the activation gating system of slow sodium $Na_V1.8$ channels (Z_{eff}) is an important quantitative parameter that determines the voltage sensitivity of the nociceptive neuron membrane. To evaluate it, the dependences of the chord conductance on transmembrane potential difference $G_{Na}(E)$ were constructed:

$$G_{Na}(E) = I_{max}(E)/(E - E_{Na}),$$

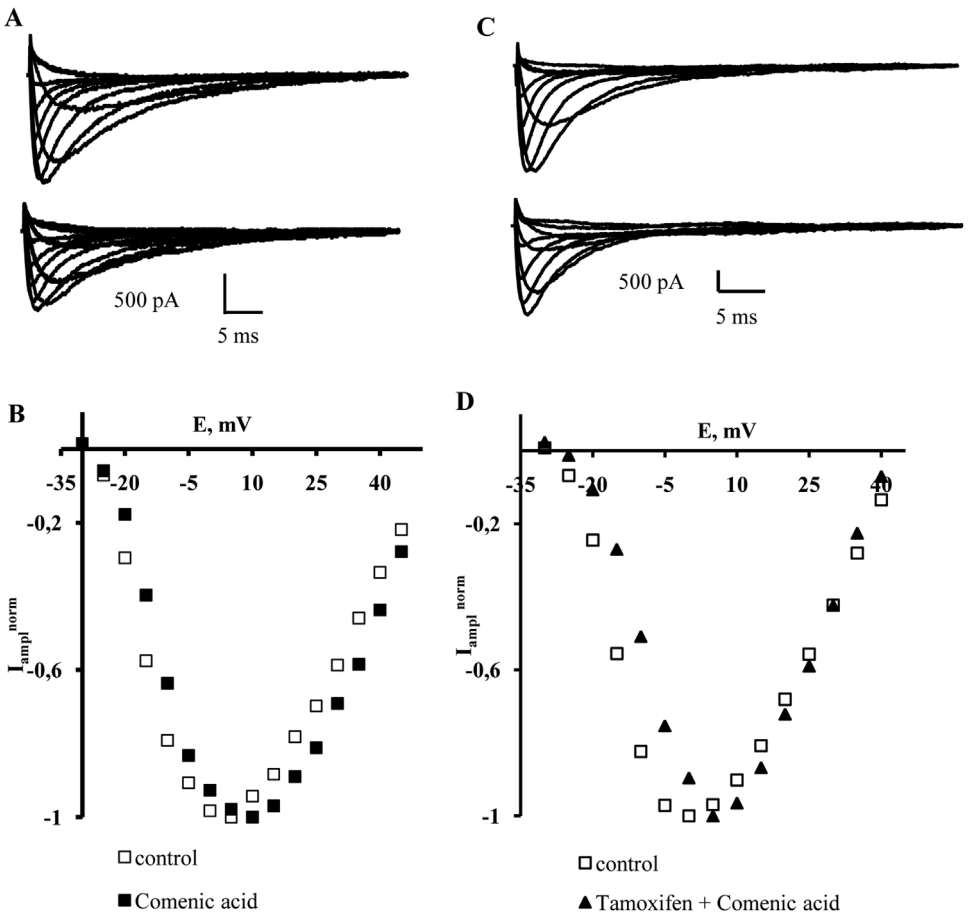

Fig. 8. Effect of comenic acid on the voltage sensitivity of slow sodium $Na_V1.8$ channels.
A, Families of slow sodium currents recorded before (top) and after extracellular application of comenic acid (100 nM).
B, Normalized peak current-voltage functions of slow sodium channels in the control experiment (white squares) and after application of 100 nM comenic acid (black squares).
C, Families of slow sodium currents recorded before (top) and after (bottom) extracellular application of comenic acid (100 nM) in experiments with intracellular solution containing the PKC inhibitor tamoxifen (10 μM).
D, Normalized peak current-voltage functions of slow sodium channels in the control experiment (white squares) and after application of 100 nM comenic acid (black triangles). The intracellular solution contained PKC inhibitor tamoxifen (10 μM).
The test potential was changed from −35 mV to 45 mV in steps of 10 mV for all records. The holding potential of 500-ms duration was equal to −110 mV. Capacitive and leakage currents were subtracted automatically.

where E_{Na} is the reversal potential for sodium ions and $I_{max}(E)$ is the amplitude value of sodium current at depolarizing potential E.

The $G_{Na}(E)$ function has an initial S-shaped segment, the slope of which is indicative of the distinctive features of the activation process voltage sensitivity. After construction of the normalized $G_{Na}{}^{norm}(E)$ function (Fig. 9), the changes in this slope after application of CA are readily observed in experiments with both standard intracellular solutions (Fig. 9A) and solutions containing tamoxifen (Fig. 9B).

The Boltzmann distribution is regularly used to evaluate the $G_{Na}(E)$ function. The stationary characteristics of transitions between the states of the sodium channel activation gating system are assumed in this case to be determined by the voltage dependence of the chord conductance. To evaluate the Z_{eff} of the activation gating system, we implemented a different approach first suggested by the authors of the membrane ionic theory [25] and later modified [24]. Fig. 10 illustrates the methodology of this Z_{eff} evaluation. The tangents of the slopes of the asymptotes passing through the first three points determine the limiting logarithmic sensitivity of $Na_V1.8$ channels to transmembrane potential change [24]. The Z_{eff} value decreased from 6.7 electron charge units in a control experiment to 4.7 electron charge units after application of CA when the standard intracellular solution was used (Fig. 10A), and from 7.0 to 4.8 when tamoxifen was present in the intracellular solution (Fig. 10B).

The average effective charge values in experiments with the standard intracellular solution, after application of CA at 100 nM, and after application of CA at 100 nM with tamoxifen in the intracellular solution, are presented in Fig. 11. It is clearly apparent that the Z_{eff} value decreases after CA was applied for both standard and tamoxifen-containing solutions. The results obtained indicate that activation of the sensory neuron membrane opioid-like receptor by CA decreases the $Na_V1.8$ channel voltage sensitivity, and that PKC does not participate in this process. The above-described radial cascade process that moves towards the neuron genome studied in organotypic tissue culture, which involves PKC, is distinct from the tangential receptor-mediated signal transduction along the neuron membrane to the $Na_V1.8$ channels [26]. The signal divergence occurs after it reaches the Na,K-ATPase/Src complex. For this reason, the units located further along the downstream signaling cascade are hardly capable of affecting the $Na_V1.8$ channel voltage sensitivity.

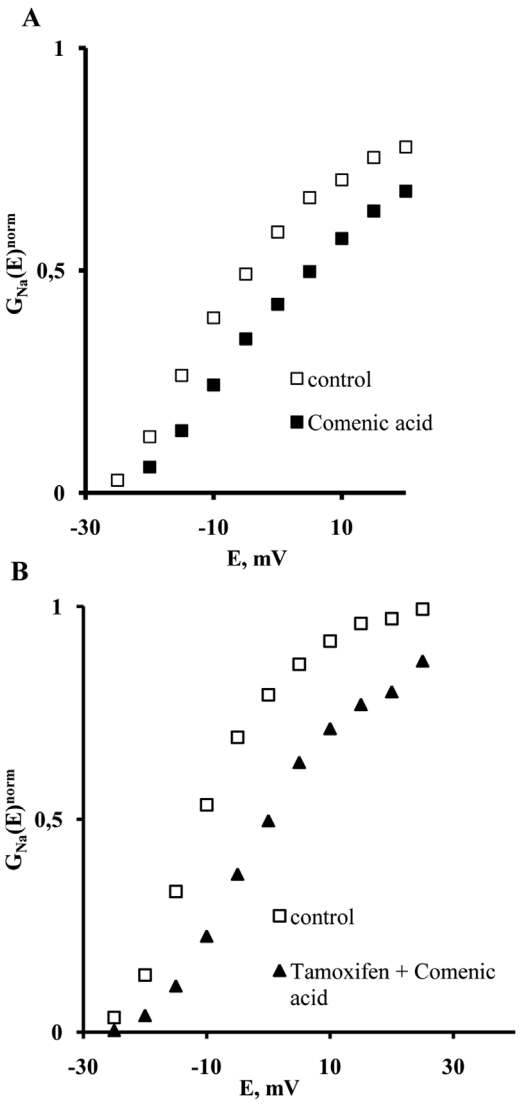

Fig. 9. Voltage dependences of chord conductance of slow sodium channels.
The $G_{Na}(E)$ function was normalized, i.e. $G_{Na}(E)^{norm} = G_{Na}(E)/G_{Na}{}^{max}(E)$ was plotted, where $G_{Na}{}^{max}(E)$ is the maximum value of the $G_{Na}(E)$ function.
A, Voltage dependence obtained using standard solutions in the control experiment (white squares) and after application of 100 nM comenic acid (black squares).
B, Voltage dependence obtained using the PKC inhibitor tamoxifen (10 μM) in the intracellular solution in the control experiment (white squares) and after application of 100 nM comenic acid (black triangles).

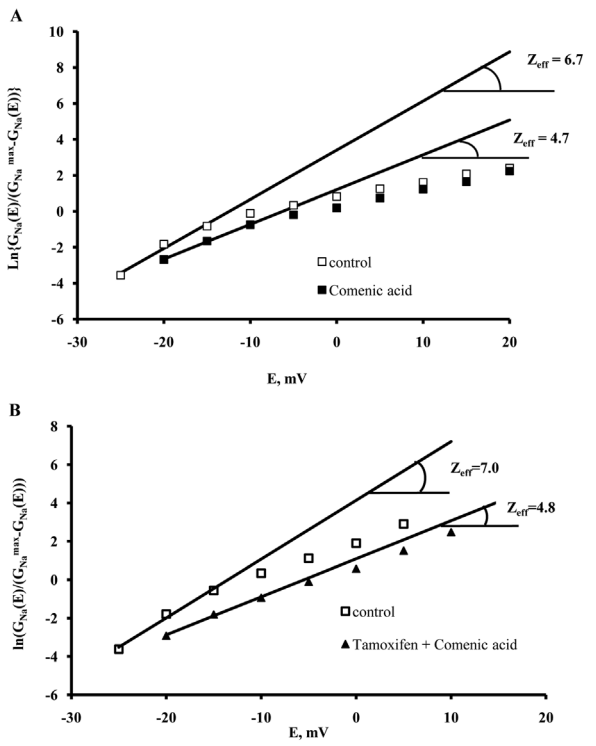

Fig. 10. Effect of comenic acid on the effective charge of the slow sodium $Na_V1.8$ channel activation gating system. Z_{eff} can be evaluated from the tangents of the slopes of the asymptotes to the initial segments of the logarithmic exponential functions (on the ordinate axis).
A, Control experiment (white squares) and data obtained after application of 100 nM comenic acid (black squares) when the standard solutions were used.
B, Control experiment (white squares) and data obtained after application of 100 nM comenic acid (black triangles) when the intracellular solution contained the PKC inhibitor tamoxifen (10 μM).

4. Discussion

CA is the active principal in the non-opioid analgesic Anoceptin®, which we have developed and which has now successfully passed the first phase of clinical trials. In addition to its principal function of relieving neuropathic pain, another remarkable feature of this agent is its ability to stimulate neurite growth (Fig. 2). CA promotes the growth of nerve fibers and the regeneration of neuronal connections, which is particularly important for the correction of a number of pathologies.

Upon binding to opioid-like receptors, CA triggers two events: one of these is directed at the $Na_V1.8$ channels along the neuron membrane, and the other one is directed at the cell genome. The signal transducer in both processes is the Na,K-ATPase/Src complex; G proteins do not participate in this signal transduction [2,5]. The signal divergence occurs after the complex. The tangential signal is transferred to the activation gating system of the $Na_V1.8$ channels, decreasing their voltage sensitivity. PKC is not involved in the signaling pathway from the opioid-like receptor to the $Na_V1.8$ channels (Fig. 10). The radial intracellular signaling pathway involves PKC, and activation of this pathway leads to the highly cell-specific downstream consequences that are manifested at the tissue level in the modulation of neurite growth (Fig. 5). PKA does not take part in signal transduction to the cell genome; its selective inhibitor KT5720 has no effect on neurite growth upon its combined application with CA (Fig. 4).

Our research has also shed some light on the further stages of the downstream cascade process in primary sensory neurons. The p38 MAP kinase signaling pathway is not involved in the signal transduction triggered by CA, as its selective inhibitor SB239063 does not block the neurite growth induced by the agent (Fig. 6). However, a representative of another major intracellular signaling pathway, ERK1/2, was observed to participate in CA-triggered signal transduction. Specific inhibition of this kinase blocks the neurite-stimulating effect of CA (Fig. 7).

Our results are summarized in the scheme presented in Fig. 12. Two important issues should be considered: first, the divergence of the tangential and radial signals triggered by CA occurs after the initial signal reaches the Na,K-ATPase/Src complex. Second, ERK1/2 was found to be involved in the downstream signaling, which suggests that it is the Na,K-ATPase-Src-ERK1/2 pathway that is activated upon binding of CA to the opioid-like receptor.

Acknowledgments

The Russian Foundation for Basic Research funded this work (Project No. 18-015-00079). The study was supported financially by

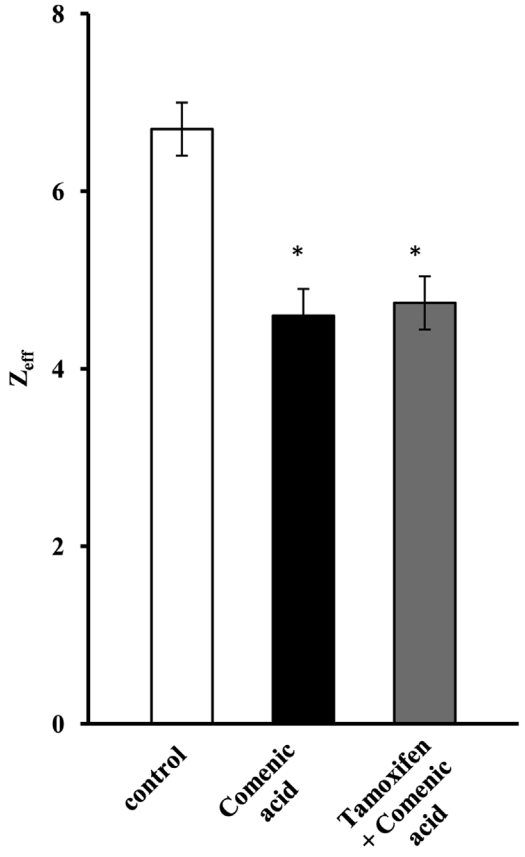

Fig. 11. Decrease of the effective charge of the slow sodium $Na_V1.8$ channel activation gating system after application of comenic acid (100 nM) when standard solutions were used and when the intracellular solution contained PKC inhibitor tamoxifen (10 μM).
Significant differences ($p < 0.05$) are indicated with asterisks.

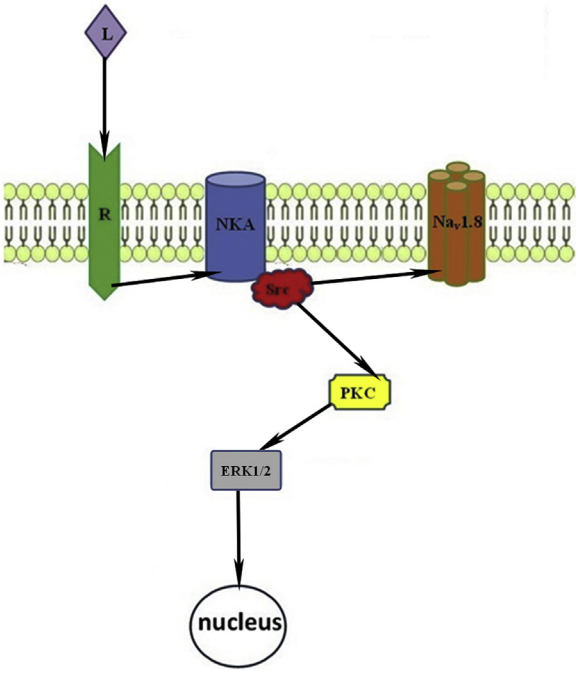

Fig. 12. Scheme of receptor-mediated modulation of intracellular signaling in sensory neurons triggered by comenic acid in tangential and radial directions.
L, comenic acid; R, opioid-like receptor; NKA, Na,K-ATPase; $Na_V1.8$, $Na_V1.8$ channel; Src, Src kinase; PKC, protein kinase C; ERK1/2, extracellular signal-regulated kinases 1/2.

the Program of Fundamental Scientific Research in State Academies for 2014–2020 (GP-14, section 64).

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