ORIGINAL ARTICLE

Src kinase is involved in comenic acid-triggered signaling pathways in sensory neurons

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Abstract

OBJECTIVES: The effects of activation of the signaling cascades triggered by binding of comenic acid to the opioid-like receptor are studied. A particular attention is paid to investigation of probable involvement of the Na,K-ATPase/Src complex in signal transduction in living sensory neurons.

METHODS: Signaling pathways activated by comenic acid are examined using organotypic tissue culture and patch-clamp techniques.

RESULTS: Binding of comenic acid to the opioid-like receptors triggers at least two signaling cascades, one of which evokes a neurite-stimulating effect and the other modulates $Na_V1.8$ channels in sensory neurons. Neurite-stimulating effect of comenic acid resulting from receptor-mediated activation of a pathway that involves Na,K-ATPase as the signal transducer is directly opposite at the tissue level to neurite-inhibiting effect produced by transducer-mediated activation of the Na,K-ATPase signaling function by nanomolar (endogenous) concentrations of ouabain. This stimulating effect is blocked by PP2, a selective Src kinase inhibitor. PP2 is also demonstrated to be involved in modulation of $Na_V1.8$ channels, which is manifested in the control of the current-voltage function.

CONCLUSIONS: Receptor- and transducer-mediated activation of the Na,K-ATPase transducer function trigger different downstream signaling pathways converging on the cell genome, which indicates that Na,K-ATPase is the unit responsible for the signal divergence. Tangential pathways along the sensory neuron membrane which modulate $Na_V1.8$ channels also involve activation of Na,K-ATPase as the signal transducer either by the opioid-like receptor-coupled or by the transducer-coupled mechanism. A considerable heterogeneity of Na,K-ATPase population suggests that different Na,K-ATPase isoforms participate in signaling cascades depending on the structure and/or function of nerve cells. Organotypic nerve tissue culture and patch-clamp investigations demonstrate that the Na,K-ATPase/Src complex operates in intracellular signaling pathways activated by comenic acid by the opioid-like receptor-coupled mechanism.

Abbreviations:
dorsal root ganglion (DRG); phosphate-buffered saline (PBS); fetal bovine serum(FBS); area index (AI); naltrexone (NTX), a nonspecific antagonist of opioid- and opioid-like receptors.

Introduction

Discovery of $Na_V1.8$ channels has opened a new prospect to study the mechanisms of nociception (Gold *et al* 1996; Kostyuk *et al* 1981, 2001). A remarkable feature of these channels is the ability to be modulated by binding of various endogenous and exogenous agents to membrane receptors coupled to $Na_V1.8$ channels, which is exemplified by the opioid-like receptor → Na,K-ATPase → $Na_V1.8$ channel signaling cascade (Krylov *et al* 2000, 2017), where Na,K-ATPase serves as the signal transducer. The three units involved in the described membrane pathway are potential targets for novel analgesics. Investigation of this mechanism of nociceptive signal modulation is of major importance not only for fundamental physiology but also for clinical medicine. It is necessary to clarify the roles played by each of the membrane proteins participating in the opioid-like receptor-mediated signaling cascades in sensory neurons. A particular attention should be paid to the membrane and submembrane processes involving Na,K-ATPase/Src complexes. Indeed, it is known that Na,K-ATPase-mediated Src-regulated signaling pathways control the cell growth in different tissues (Lai *et al* 2013), and this mechanism is poorly studied in sensory neurons.

Comenic acid (5-hydroxy-γ-pyrone-2-carboxylic acid) is a derivative of γ-pyrone (Figure 1). The starting studies of probable molecular mechanisms of γ-pyrones targeting were inspiring: two agents effectively decreased the voltage sensitivity of $Na_V1.8$ channels (Derbenev *et al* 2000). Results of the further research on the role of γ-pyrone derivatives in nociception are summarized elsewhere (Krylov *et al* 2017). In this review, it is demonstrated that extracellular application of comenic acid leads to a very pronounced decrease in the voltage sensitivity of $Na_V1.8$ channels due to activation of the opioid-like receptor-coupled membrane signaling mechanism. Indeed, a nonspecific antagonist of opioid and opioid-like receptors naltrexone (NTX) blocked the effect of comenic acid. Also, combined application of comenic acid and ouabain (200 μM) did not significantly affect the $Na_V1.8$ channel voltage sensitivity. At this relatively high concentration, ouabain totally inhibits both pumping and transducer functions of Na,K-ATPase, thus interrupting the tangential signal transduction along the neuron membrane triggered by binding of comenic acid to the opioid-like receptor and directed to $Na_V1.8$ channels. Moreover, comenic acid was shown to be comparable to morphine in the efficiency to modulate $Na_V1.8$ channels (Derbenev *et al* 2000; Krylov *et al* 2000, 2017). The agent switches on the three background mechanisms: reduces the density of the channels, positively shifts the $Na_V1.8$ channel activation gating process along the voltage axis, and decreases effective charge transfer (Z_{eff}) of the activation gating system (in other words, decreases the $Na_V1.8$ channel voltage sensitivity). The latter process is dose-dependent, displaying binding of comenic acid to the opioid-like receptor characterized by K_d=100 nM and the Hill coefficient n=0.5.

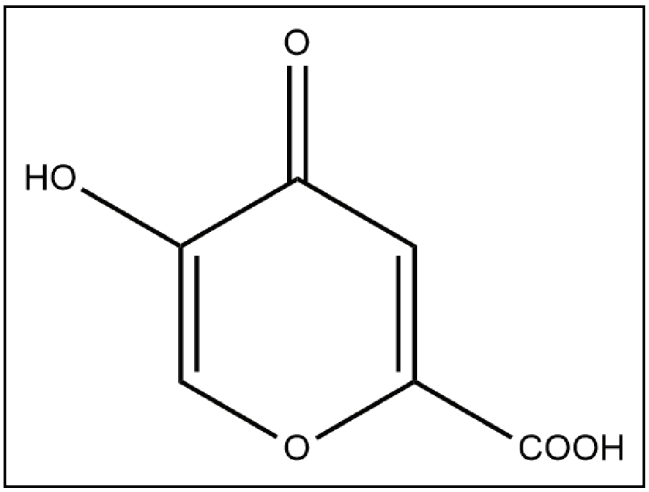

Fig. 1. Structural formula of comenic acid.

Preclinical (Derbenev *et al* 2000; Plakhova *et al* 2014) and clinical (Lopatina & Polyakov 2011) trials of comenic acid have revealed an important feature of this substance: it does not exhibit any addictive properties and other adverse side effects upon administration both in humans and animals, which suggests that the agent does not interact with μ-opioid receptors and should be absolutely safe for clinical application. Up to now, only comenic acid, several closely related γ-pyrones and morphine are demonstrated to specifically activate the opioid-like receptor coupled to $Na_V1.8$ channels, but the affinity of morphine to this receptor is higher than that of comenic acid (K_d = 8 and 100 nM, respectively) (Derbenev *et al* 2000; Krylov *et al* 2000). Very importantly, endogenous μ-opioid agonists endomorphin-1 and endomorphin-2 (Zadina *et al* 1997) do not display any $Na_V1.8$ channel-modulating activity (Katina *et al* 2003), which indicates that not every potent agonist of μ-opioid receptors is capable of activating the opioid-like receptor.

The effects of activation of the signaling cascades triggered by binding of comenic acid to the opioid-like receptor will be studied in the present work at two levels: at the tissue level by organotypic tissue culturing and at the membrane level by the patch-clamp method. A particular attention will be paid to investigation of probable involvement of the Na,K-ATPase/Src complex in signal transduction in living sensory neurons.

Methods

Organotypic nerve tissue culture

The effects of comenic acid, ouabain and Src kinase inhibitor PP2 on neurite growth of 10–12-day old chicken embryo sensory ganglia were studied in organotypic tissue culture as described in detail previ-

ously (Lopatina *et al* 2012). The *White Leghorn* chicken embryos provided by Sinyavino Poultry (Leningrad Region, Russia) were used. Experiments were performed on explants cultured in collagen-coated Petri dishes in CO_2 incubator (Sanyo, Japan) at 36.5 °C and 5% CO_2 for three days. The culture medium contained 45% Hanks' solution and 40% Eagle's medium supplemented with insulin (0.5 U/ml), glucose (0.6%), glutamine (2 mM), gentamicin (100 U/ml), 5% chick embryo extract, and 10% fetal bovine serum (FBS). Explants cultured without exposure to the test substance served as control. NTX, ouabain, and PP2 (all from Sigma, USA) were added to the culture medium at 10 mM, 10 nM, and 10 μM, respectively, whereas comenic acid was added at 10 nM.

During the first day of embryonic nerve tissue culturing, explants spread over the collagen substrate; neurite growth and eviction of proliferating and migrating cells begins. After three days of culturing, two distinct zones can be recognized both in control and experimental sensory ganglia explants. The central zone is composed of nonmigrating differentiating neuroblasts, and the peripheral zone (also termed as the growth zone) consists of fibroblast-like cells, glia and growing neurites; synaptic connections are not yet formed.

Neurite growth was evaluated quantitatively by the morphometric method. The area index (AI) was calculated as the ratio of the explant growth area to the area of the central zone of a ganglion. AI of control explants was taken for 100%.

Laser scanning confocal microscope (LSM-710; Carl Zeiss, Germany) was integrated with Axio Observer Z1 inverted optical microscope (Carl Zeiss, Germany). Explants were rinsed in phosphate-buffered saline (PBS; Sigma, USA) and fixed for 3 min with 4% formalin in PBS. After washing with PBS, explants were permeabilized with 0.3% Triton X-100 in PBS for 15 min and blocked with PBS containing 10% FBS at room temperature for 30 min. Explants were then incubated with primary anti-Neurofilament 200 antibody (Sigma, USA) overnight at 4 °C, washed and incubated with FITC conjugated secondary antibody for 2 h at 37 °C, and further analyzed with ImageJ and ZEN_2012 (Carl Zeiss, Germany) software. Experiments were carried out using the equipment of the Confocal Microscopy Collective Use Center (Pavlov Institute of Physiology of the Russian Academy of Sciences).

Patch-clamp technique

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. The animals were obtained from the Biocollection of Pavlov Institute of Physiology supported by the Russian Federal Agency of Research Organizations.

Experiments were performed on short-term cultured dissociated dorsal root ganglion (DRG) neurons isolated from newborn *Wistar* rats. These nociceptive cells are small dark neurons with high density of $Na_V1.8$ channels (Djouhri *et al* 2003). Dorsal ganglia were isolated from the L_5–S_1 region of the spinal cord and placed in Hank's solution. Enzymatic treatment (Kostyuk *et al* 1975) was performed for 8 min at 37 °C in the solution containing 1 ml Hank's solution, 1 ml Eagle's medium, 2 mg/ml type 1A collagenase, and 1 mg/ml pronase E. The buffer used was 1 mM HEPES Na, pH 7.4. After this procedure, the ganglia were thoroughly washed by centrifugation (1 min, 900 rpm) with washing the supernatant solution. Both washing and cultivation were performed using the solution consisting of Eagle's medium with glutamine based on Earle's solution (1:1), 10% FBS, glucose (0.6%), and gentamicin (40 U/ml). Mechanical dissociation was carried out by pipetting. The culture fluid was added to the resulting cell suspension to obtain the desired cell density in a plastic Petri dish. Non-neuronal cells were removed by allowing them to settle onto the surfaces of plastic 90-mm Petri dishes for 25 min at 37 °C, while the remaining cells (mostly, dissociated sensory neurons) were cultured on the collagen-coated surfaces of 40-mm Petri dishes.

Ion currents were recorded under the whole-cell patch-clamp configuration. The extracellular solution contained 65 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 70 mM choline chloride, 10 mM HEPES Na, and 0.1 μM tetrodotoxin, pH 7.4. The intracellular solution contained 100 mM CsF, 10 mM NaCl, 40 mM CsCl, 2 mM $MgCl_2$, and 10 mM HEPES Na, pH 7.2. Exclusion of potassium ions minimized all the components of potassium currents; the intracellular fluoride was used to block calcium currents (Kostyuk *et al* 1975). All the reagents were from Sigma, USA. Experiments were performed at room temperature of 22–24 °C and controlled by hardware-software complex consisting of EPC-7 amplifier, personal computer, and self-made software system for the automatic running of experiments. The series resistance (R_S) was constantly monitored and maintained below 2 MΩ (Osipchuk & Timin 1984). When the amplitude of the sodium current was less than approximately 1 nA, the R_S error did not exceed 2 mV. The leakage and capacitive currents were subtracted automatically.

The limiting-slope procedure (Almers 1978) is the approach used in the present work to estimate Z_{eff}, as the characteristics of activation and inactivation gating devices of $Na_V1.8$ channels are justified to be perfectly suited for application of the Almers' method (Krylov *et al* 2000, 2017; Yachnev *et al* 2012). The ratio of the number of open channels (N_o) to the number of closed channels (N_c) is calculated as

$$N_o/N_c = G_{Na}(E)/[G_{Na}{}^{max} - G_{Na}(E)],$$

where G_{Na}^{max} and $G_{Na}(E)$ are the maximal value and the voltage dependence of the chord conductance, respectively. $G_{Na}(E)$ can be obtained in patch-clamp experiments as

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

where I_{max} is the amplitude value of sodium current, E_{Na} is the reversal potential for sodium ions. $G_{Na}(E)$ is a monotonous function approaching its maximum G_{Na}^{max} at positive E. According to the Almers' theory, the limiting-slope procedure can be applied:

$$\lim_{E\to-\infty}(N_o/N_c) = \lim_{E\to-\infty}\{G_{Na}(E)/[G_{Na}^{max} - G_{Na}(E)]\} \to C\cdot\exp[(Z_{eff}e_0E)/(kT)], \quad E\to-\infty \text{ (Equation 1)}$$

where N_o is the number of open channels, N_c is the number of closed channels when the membrane potential E approaches minus infinity ($E\to -\infty$). Z_{eff} can be estimated from the slope of the asymptote passing through the first points determined by the very negative values of E (Equation 1), since the Boltzmann's principle is applicable at these potentials, where k is the Boltzmann constant, T is the absolute temperature, C is a constant, e_0 is the electron charge.

When the chord conductance dependencies are obtained, the Almers' limiting-slope procedure (Equation 1) can be applied, making it possible to evaluate Z_{eff} by constructing the voltage dependence of the logarithmic voltage sensitivity function $L(E)$:

$$L(E) = ln\,(G_{Na}(E)/(G_{Na}^{max} - G_{Na}(E)) \quad \text{(Equation 2)}$$

The asymptote passing through the first points of the $L(E)$ function obtained at the most negative E allows to calculate Z_{eff}, which is linearly proportional to the tangent of the asymptote slope.

Statistical analysis

The data were analyzed with STATISTICA 10.0 and expressed as the mean value ± standard error of the mean. Statistical significance was set at $p<0.05$.

Results

Organotypic nerve tissue culture

Comenic acid is demonstrated to exhibit a pronounced neurite-stimulating effect at the tissue level (Figure 2). At 10 nM, the agent significantly stimulated growth of sensory ganglia neurites by 58±6% (n = 30, $p<0.05$) (Figure 3). Comenic acid was earlier shown by the patch-clamp method to activate the opioid-like receptor (Krylov *et al* 2017), so NTX was used here to question the role of this receptor in neurite-stimulating action of comenic acid. Addition of NTX at 10 mM to the culture medium resulted in a significant inhibition of neurite growth with AI by 42±6% (n=28, $p<0.05$) below control (n=25) (Figure 3). Neurite-stimulating effect was not observed upon combined application of comenic acid (10 nM) and NTX (10 mM) with AI by 19±8% (n=30) lower than control (n=27). These data indicate that neurite-stimulating effect of comenic acid is due to activation of the opioid-like receptor, which signals through Na,K-ATPase as the transducer (Krylov *et al* 2017).

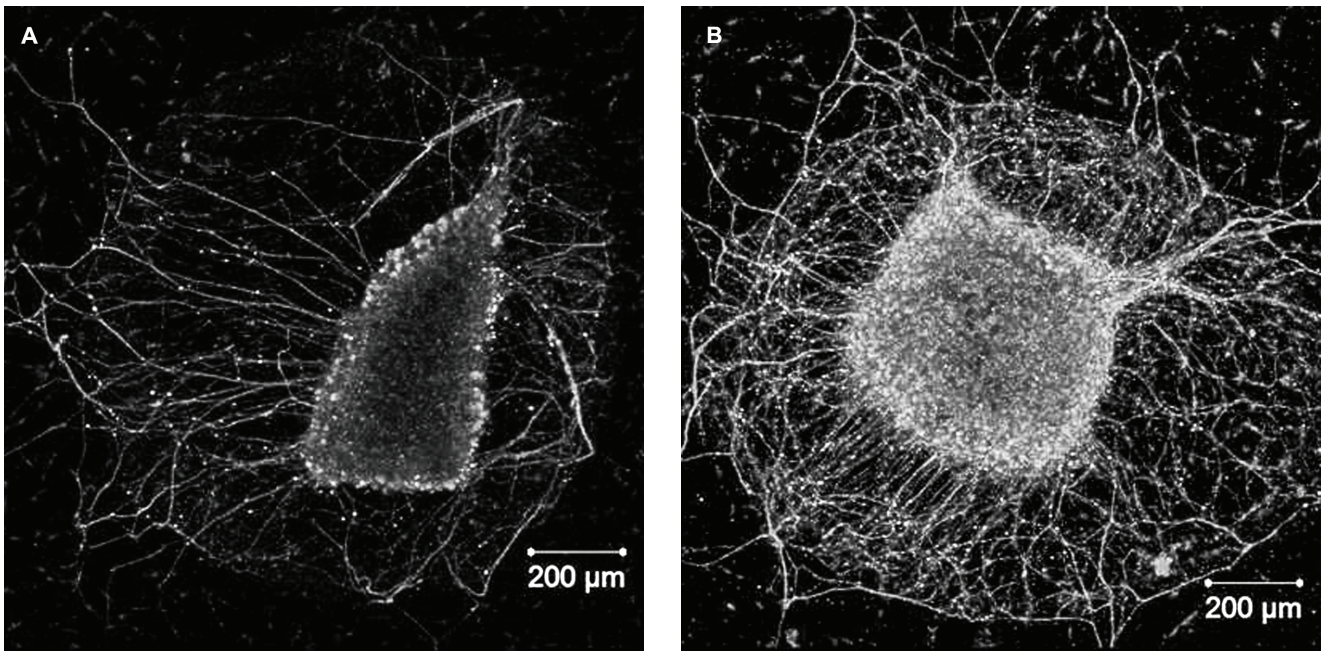

Figure 2. Imaging of a sensory ganglion of 10-day old chick embryo (3rd day of culturing; LSM-710, Carl Zeiss, Germany). The ganglion is stained with anti-Neurofilament antibody. Scale bar 200 µm. **A** – control; **B** – comenic acid (10 nM)

At 10 nM and even at 0.1 nM, ouabain significantly inhibits sensory ganglia neurite growth due to activation of the Na,K-ATPase transducer function (see also: Lopatina *et al* 2012). Combined action of comenic acid (10 nM) and ouabain (10 nM) eliminated neurite-stimulating effect of comenic acid. The AI of experimental explants was 25±6% (n=29) below control (Figure 4). An uncertain stimulation of neurite growth was observed upon culturing in the medium containing both comenic acid (10 nM) and ouabain (0.1 nM) with AI by 20±6% (n=30) above control (Figure 4), whereas ouabain alone at the same concentration inhibited the neurite growth by 50±7% (n=25, $p<0.05$). Since combined application of growth-stimulating comenic acid and growth-inhibiting ouabain yields the AI values close to the arithmetical sum of the effects of single agents, two largely independent signaling pathways triggered by activation of two distinct membrane targets (the opioid-like receptor and Na,K-ATPase as the signal transducer) and converging on the cell genome are likely to exist in sensory neurons. When the ouabain concentration is decreased from 10 nM to 0.1 nM, while the concentration of comenic acid remains at 10 nM, the effect of ouabain alone and the effect of combined application of the two agents display a fairly parallel shift (Figure 4), which strongly supports this idea.

Culturing of the sensory ganglia in the medium containing comenic acid (10 nM) and Src kinase inhibitor PP2 (10 μM) resulted in elimination of neurite-stimulating effect (Figure 5), thus indicating that Src kinase is also involved as a consecutive unit in the intracellular signaling pathways activated by comenic acid by the receptor-coupled mechanism.

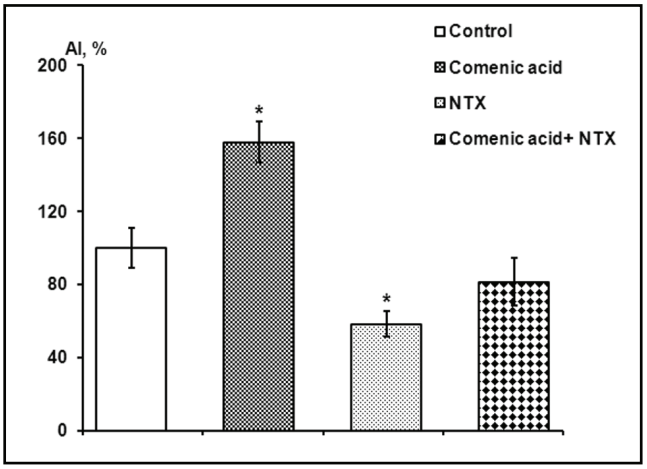

Fig. 3. Naltrexone inhibits the effect of comenic acid on neurite growth. Effect of comenic acid (10 nM), NTX (10 mM), and their combined application on sensory ganglia neurite growth. The ordinate shows the area index (AI, %) of explants. Data are expressed as mean ± SEM. Significant differences ($p<0.05$) are indicated with asterisks.

Patch-clamp technique

It is noteworthy that binding of comenic acid to the opioid-like receptor decreases the $Na_V1.8$ channel voltage sensitivity, *i.e.*, the value of effective charge transfered in the activation gating system of these channels (Z_{eff}) is strongly reduced (Derbenev *et al* 2000; Plakhova *et al* 2014). In addition to that, the decaying branch of the current-voltage function strongly shifts along the *E* axis in the depolarizing direction, which also leads to the decrease of the sensory neuron excitability (Krylov *et al* 2017). Involvement of Src kinase in these mechanisms is studied in the present work.

The $Na_V1.8$ currents were recorded using the intracellular solution containing Src kinase inhibitor PP2 at 10 μM. These recordings were regarded as control

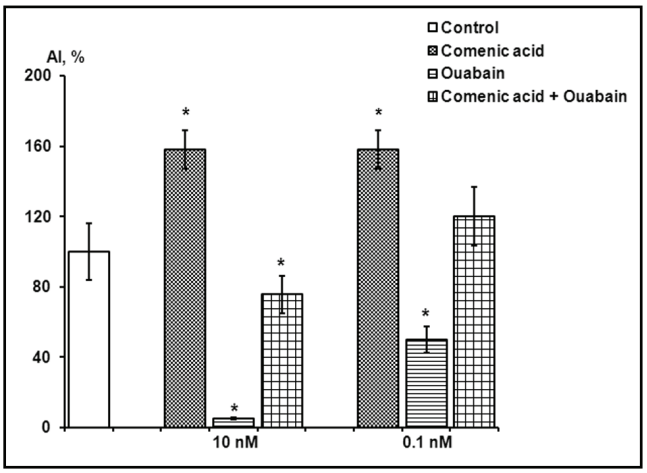

Fig. 4. Combined application of comenic acid (10 nM) and ouabain (10 nM/0.1 nM) results in the decrease of comenic acid-induced neurite growth. The ordinate shows the area index (AI, %) of explants. Data are expressed as mean ± SEM. Significant differences ($p<0.05$) are indicated with asterisks.

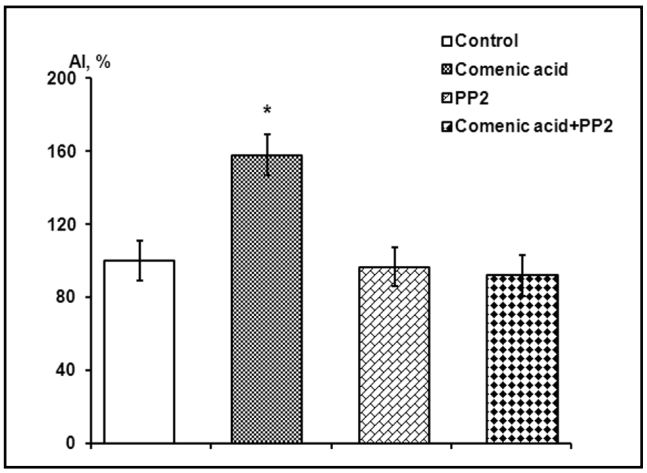

Fig. 5. PP2 blocks the effect of comenic acid on neurite growth. Comenic acid (10 nM) increases sensory ganglia neurite growth by 58%. Src kinase inhibitor PP2 (10 μM) alone and comenic acid (10 nM) in the presence of PP2 (10 μM) do not affect neurite growth. The ordinate shows the area index (AI, %) of explants. Data are expressed as mean ± SEM. Significant differences ($p<0.05$) are indicated with asterisks.

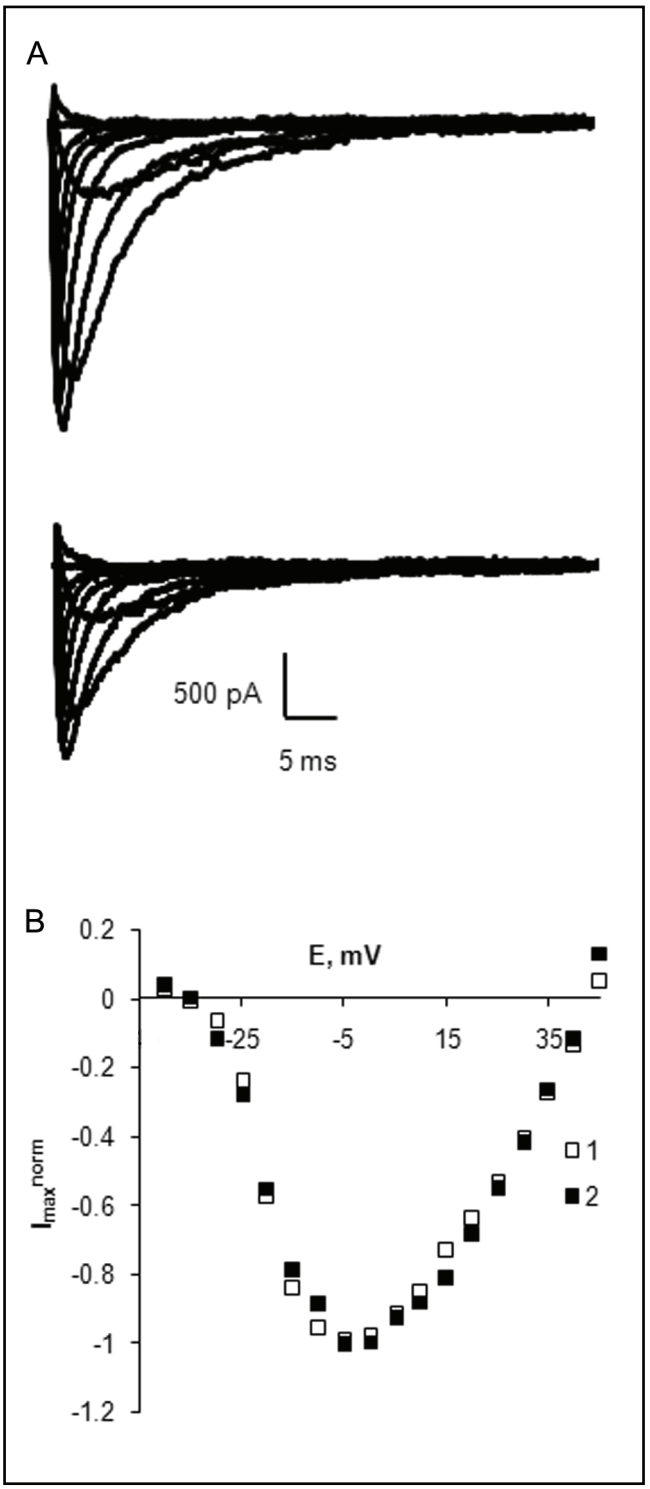

Fig. 6. Responses of $Na_V1.8$ channels on intracellular application of Src kinase inhibitor PP2 (control data) and on combined application of comenic acid in the extracellular solution with PP2 in the intracellular solution (experimental data). **A.** Families of sodium currents recorded in the control experiment containing PP2 at 10 μM in the intracellular solution (top) and after extracellular application of comenic acid at 100 nM on the background of PP2 at 10 μM in the intracellular solution (bottom). The test potential was changed from –35 mV to 45 mV with a step of 10 mV. The holding potential of 500-ms duration was equal to –110 mV in all records. **B.** The normalized maximal current-voltage function after intracellular application of PP2 at 10 μM (control data, 1) and after extracellular application of comenic acid at 100 nM on the background of PP2 at 10 μM in the intracellular solution (experimental data, 2).

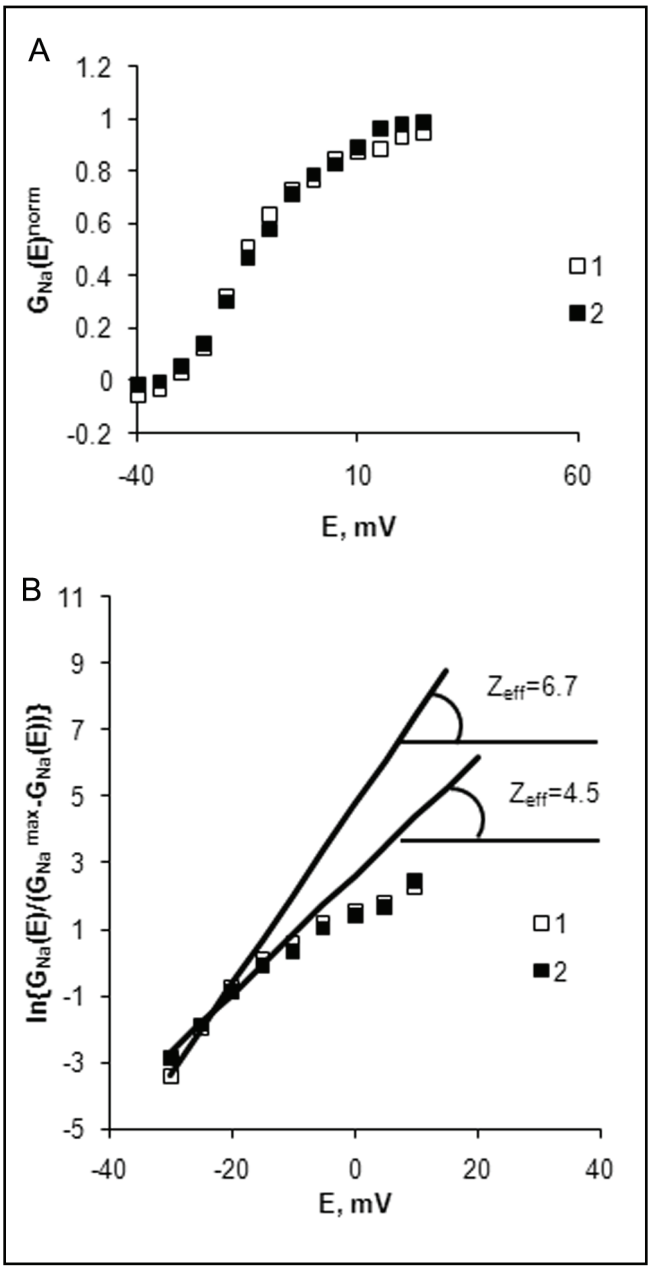

Fig. 7. Decrease of the effective charge of $Na_V1.8$ channel activation gating device after combined application of comenic acid and PP2. **A.** Voltage dependence of the normalized maximal conductance after intracellular application of PP2 at 10 μM (control data, 1) and after extracellular application of comenic acid at 100 nM on the background of PP2 at 10 μM in the intracellular solution (experimental data, 2). **B.** Z_{eff} evaluation by the Almers' limiting-slope procedure after intracellular application of PP2 at 10 μM (control data, 1) and after extracellular application of comenic acid at 100 nM on the background of PP2 at 10 μM in the intracellular solution (experimental data, 2).

and compared with experimental recordings of ion currents arising in response to extracellular application of comenic acid at 100 nM on the background of PP2 in the intracellular solution. The families of $Na_V1.8$ currents in control experiments and after application of comenic acid are presented in Figure 6A.

It is clearly seen that the amplitude values of the currents are slightly decreased, which can find its partial explanation in the "run-down" effect inherent to the patch-clamp method (Hamill *et al* 1981; Sakmann & Neher 2009). The normalized peak current-voltage function of an exemplary neuron is displayed in Figure 6B, and its decaying branch demonstrates no shift along the *E* axis after the experimental solution has been applied. The voltage dependencies of normalized $G_{Na}(E)$ functions differ between control and experimental data at negative *E* (Figure 7A). When $G_{Na}(E)$ dependencies are obtained, the Almers' limiting-slope procedure (Equation 2) can be applied, making it possible to evaluate Z_{eff} at the very negative potentials *E* (Figure 7B). After extracellular application of the experimental solution, a decrease in Z_{eff} from the control value of 6.7±0.3 (n=24) to 4.6±0.4 (n=22) is observed due to activation of the comenic acid-triggered receptor-coupled membrane signaling mechanism (Figure 7B).

So, effective charge transfer of the $Na_V1.8$ channel activation gating system is not influenced by the Src kinase inhibitor. But PP2 strongly affects another important gating characteristics: all our experimental recordings (n=22) demonstrate that its intracellular application totally compensates for the positive shift of the current-voltage function (Figure 6B), which is a phenomenon inherent to activation of the opioid-like receptor by comenic acid (Derbenev *et al* 2000; Krylov *et al* 2017; Plakhova *et al* 2014). The current-voltage function remained unshifted after combined application of comenic acid and PP2 (Figure 6B). In our additional experiments, both intracellular and extracellular application of PP2 alone was not shown to affect this function. It is thus demonstrated that PP2 is involved in modulation of $Na_V1.8$ channels, which is manifested in the control of the current-voltage function, and this mechanism strongly regulates the sensory neuron excitability (Krylov *et al* 2017). The data obtained suggest that Src kinase might be another intermediate unit in the studied tangential membrane signaling cascade, consecutive to both the opioid-like receptor and Na,K-ATPase.

Discussion

The main finding of the present work is the detection at the tissue level of neurite-stimulating effect of comenic acid resulting from receptor-mediated activation of the signaling pathway that involves Na,K-ATPase as the signal transducer. On the contrary, transducer-mediated activation of the Na,K-ATPase transducer function by nanomolar (endogenous) concentrations of ouabain inhibits neurite growth (Lopatina *et al* 2012), which indicates that the receptor- and transducer-mediated signaling pathways converging on the sensory neuron cell genome are different. It is most probably Na,K-ATPase that is the unit responsible for signal divergence. A considerable heterogeneity of Na,K-ATPase population suggests that different Na,K-ATPase isoforms should be involved in signaling depending on the structure and/or function of nerve cells.

The situation is substantially complicated, as there are four known Na,K-ATPase α subunit isoforms, and their expression is very cell type- and tissue-specific. The α1 subunit is essentially ubiquitous. Animal hearts express *α*1, while it varies between species if *α*2 and/or *α*3 can be also detected (Henriksen *et al* 2013). In human hearts, all three isoforms are expressed (Schwinger *et al* 1999). In adult vertebrates, kidney cells express mostly α1 (Wetzel & Sweadner 2001), muscle and glial cells – α1 and α2 (He *et al* 2001), sperm cells – α1 and α4 isoforms (Jimenez *et al* 2011). Neurons may express α1, α2, and α3 isoforms, and the neuronal type is suggested to be the determining factor (Peters *et al* 2001; Dobretsov & Stimers 2005; Bøttger *et al* 2011). Most neurons co-express two Na,K-ATPase isoforms, the ubiquitous *α*1 and the more selectively expressed α3 (Azarias *et al* 2013). Regardless of cell size, the α1 isoform was found in DRG neurons, and only 16% of them expressed the α3 isoform (Dobretsov *et al* 1999).

Investigation of the ability of different Na,K-ATPase isoforms to perform the transducer function in sensory neurons is a task for the further research. It is sufficient to observe that the isoform population is rather heterogeneous, which provides an explanation for the results obtained here.

The non-pumping function of Na,K-ATPase was firstly discovered in cardiomyocytes. This protein was demonstrated to act also as a signal transducer by relaying the message of its interaction with nanomolar concentrations of extracellular ouabain to the nucleus through multiple interconnected gene regulatory pathways, some of which have been identified (Kometiani *et al* 1998; Haas *et al* 2000). At the same time, the Na,K-ATPase signaling function was independently found by us in the membrane of DRG neuron (Krylov *et al* 2000; see also Krylov *et al* 2017). We have predicted that $Na_V1.8$ channels responsible for coding of nociceptive information could be modulated due to two novel targeting mechanisms. The first one is activation of the opioid-like receptors; the second, activation of the Na,K-ATPase transducer function. Development of analgesics triggering these mechanisms should lead in the near future to successful relief of several chronic pain manifestations. Any endogenous or exogenous molecule that specifically activates the opioid-like receptor at physiologically adequate conditions can be potentially considered as a medicine. Our assumption that comenic acid effectively and specifically binds to this receptor can account for the success of preclinical trials and the first phase of clinical trials of novel analgesic Anoceptin® containing the agent as the medicinal substance (Derbenev *et al* 2000; Lopatina & Polyakov 2011; Plakhova *et al* 2014).

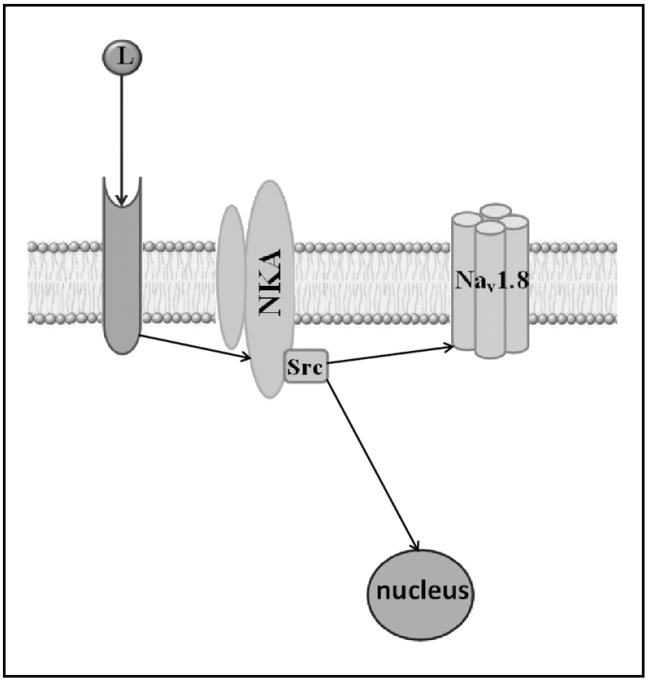

Fig. 8. Probable receptor-mediated pathways triggered by comenic acid (L) and controlled by Na,K-ATPase (NKA) and Src kinase.

Culturing of sensory ganglia in the medium containing comenic acid and Src kinase inhibitor PP2 resulted in elimination of neurite-stimulating effect of comenic acid, thus indicating that Src kinase is also included as a series unit in the intracellular signaling pathway activated by comenic acid by the receptor-coupled mechanism. This signaling pathway triggered by binding of comenic acid to the opioid-like receptor and directed to the cell genome involves a consecutive activation of Na,K-ATPase as the signal transducer and Src kinase. It is known that Na,K-ATPase-mediated Src-regulated signaling pathways control cell growth in different tissues (Lai *et al* 2013), but this mechanism is poorly studied in sensory neurons. Our data obtained by organotypic nerve tissue culture and patch-clamp methods partially fill this gap.

A PP2 effect on $Na_V1.8$ channels was also registered in the patch-clamp experiments. Src kinase inhibition does not affect the effective charge of the $Na_V1.8$ channel activation gating device, but it influences its current-voltage function in such a manner that compensates for the effect induced by application of comenic acid alone. This agent was previously demonstrated both to decrease Z_{eff} and shift the decaying branch of the current-voltage function to the right along the *E* axis (Derbenev *et al* 2000; Plakhova *et al* 2014). This positive shift results in a decrease of the nociceptive neuron excitability, thus enhancing the antinociceptive action of comenic acid (Krylov *et al* 2017). The sensory neuron excitability is also known to be controlled by the voltage dependence of the steady-state activation gating function of $Na_V1.8$ channels, which means that the negative shift of this function along the *E* axis dramatically decreases the voltage threshold of voltage-gated sodium channels and, in turn, it increases the firing frequency of sensory membrane (Akoev *et al* 1988). This mechanism is shown to facilitate the sensory neuron excitation following $Na_V1.8$ channel phosphorylation by PKA (England *et al* 1996). As the positive shift of the current-voltage function resulting from application of comenic acid is compensated by Src kinase inhibition, PP2 diminishes the efficiency of the comenic acid-triggered signaling antinociceptive pathway which is a powerful mechanism of pain relief at the organismal level (Krylov *et al* 2017).

In summary, neurite-stimulating effect of comenic acid on sensory ganglia neurite growth is detected at the tissue level. At the membrane level, the agent modulates $Na_V1.8$ channels due to activation of another signaling pathway. Both Na,K-ATPase and Src kinase are shown to be consecutively involved in the receptor-mediated signaling pathways triggered by binding of comenic acid to the opioid-like receptor (Figure 8). These pathways are also demonstrated to be largely independent from the transducer-mediated signaling cascades triggered by direct activation of the Na,K-ATPase transducer function by nanomolar concentrations of ouabain, which brings about an issue concerning the heterogeneity of Na,K-ATPase/Src complexes participating in signaling in sensory neurons. This issue will be clarified in our further studies.

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Conflict of interest

The authors declare that they have no conflict of interest.

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