ORIGINAL ARTICLE

A novel mechanism of modulation of slow sodium channels: from ligand-receptor interaction to design of an analgesic medicine

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Abstract

OBJECTIVES: Binding of gamma-pyrone derivatives to an opioid-like membrane receptor was shown to modulate the functional properties of $Na_V1.8$ channels, responsible for the nociceptive signal coding, by a novel mechanism including Na+,K+-ATPase as a signal transducer. Our goal was to establish the criteria for gamma-pyrones to activate the receptor, investigate the signaling mechanism physiologically and examine the analgesic effects possibly resulting from switching on the receptor-coupled signalization cascade.
METHODS: Action of gamma-pyrones was investigated by patch-clamp and behavioral (formalin, hot-plate and intracranial self-stimulation (ICSS)) techniques. The molecular structural information was obtained in quantum-chemical calculations.
RESULTS: Patch-clamp and quantum-chemical data indicate that gamma-pyrones interact with the opioid-like receptor in a form of Ca2+ chelate complex, thus decreasing the voltage sensitivity of $Na_V1.8$ channels. One of active molecules (comenic acid) was shown to produce an effective analgesia in hot-plate and formalin tests and induce a decrease in ICSS of the lateral hypothalamus at very low concentrations.
CONCLUSIONS: Comenic acid does not switch on the opioid-controlled signaling cascades. It activates a novel additional nociceptive opioid-like-receptor-coupled mechanism that does not involve G-proteins and functions in parallel to well-known opioidergic system. A new analgesic Anoceptin containing comenic acid as an active substance should be very effective and safe for humans. It also should not evoke negative side effects and euphoria, which was successfully verified during the first phase of clinical trials.

Introduction

A radically new prospect was opened in the development of analgesics when a novel target molecule was discovered in the sensory neuron membrane: slow sodium (tetrodotoxin-resistant) channel (Kostyuk *et al* 1981, 2001). It is well known now that $Na_V1.8$ channels are responsible for nociception in mammals (Borovikova *et al* 1997; Gold *et al* 1996; Jarvis *et al* 2007). Among various types of voltage-gated sodium channels encoded by different genes only a few may be involved in the development of pain syndromes. The

most important transcript type is SNS/PN3 (Djouhri *et al* 2003); the respective $Na_V1.8$ channels are predominantly expressed in small (capsaicin-sensitive) dorsal root ganglion (DRG) cells and trigeminal neurons, and produce sodium currents, which are distinct from the classical fast sodium currents not only by their resistance to tetrodotoxin (TTX), but also by their slower kinetics due to specific characteristics of their gating machinery. We predict that $Na_V1.8$ channels are specifically coupled to opioid-like receptors (Krylov *et al* 2000) and consequently can be modulated by derivatives of gamma-pyrone by a receptor-activated mechanism. A pronounced analgesic effect arising from receptor-coupled modulation of $Na_V1.8$ channels was detected in behavioral tests.

Methods

The 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 I.P. Pavlov Institute of Physiology of the Russian Academy of Sciences approved all experimental procedures with the animals.

Patch-clamp technique

The 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 I.P. Pavlov Institute of Physiology of the Russian Academy of Sciences approved all experimental procedures with the animals. 2.1. Patch-clamp technique. Experiments were performed on short-term cultured DRG neurons isolated from newborn Wistar rats, obtained from the vivarium of I.P. Pavlov Institute of Physiology of the Russian Academy of Sciences. These nociceptive cells are small dark neurons with high density of NaV1.8 channels (Djouhri *et al* 2003). Dorsal ganglia were isolated from the L5-S1 region of the spinal cord and were placed in Hank's solution. Enzymatic treatment (Kostyuk *et al* 1975) and ionic current recording and data processing were performed as we have described earlier (Yachnev *et al* 2012; Katina *et al* 2013). Gamma-pyrones were synthesized by Dr. I.N. Domnin (St. Petersburg State University) using the Garkusha method (Garkusha 1953). Other reagents were from Sigma. The series resistance (Rs) was constantly monitored in all the experiments and maintained below 2 MΩ (Osipchuk & Timin 1984). When the amplitude of the sodium current was less than approximately 1 nA, the series resistance error did not exceed 2 mV.

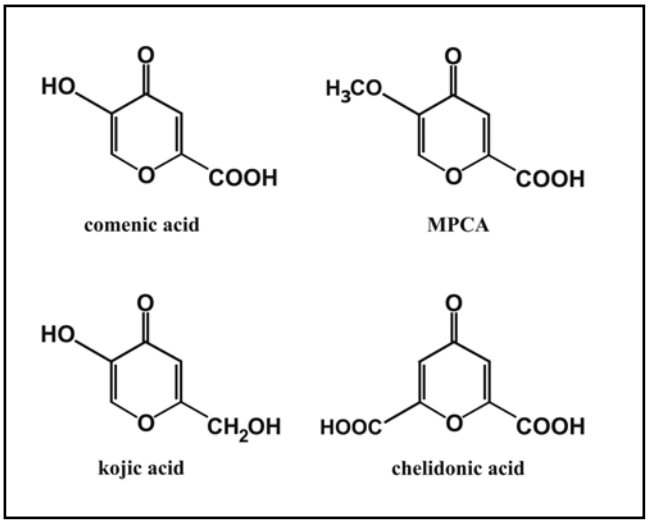

Fig. 1. Structural formulae of gamma-pyrones.

Calculational methods

A full geometry optimization of comenic acid, chelidonic acid, kojic acid and 5-methoxy-gamma-pyrone-2-carboxylic acid (MPCA) was performed by RHF method with 6-31G* basis set (Hariharan & Pople 1973) within GAMESS program package (Schmidt *et al* 1993). The structural formulae of these molecules are presented in Supporting Information Figure 1. The molecules were considered not only in the form of free acids and their anions, but also (whenever possible) their Ca^{2+} and Na^+ salts, Ca^{2+} chelates, as well as Ca^{2+} and Na^+ salts of Ca^{2+} chelates. The calculations were carried out mostly in the gas phase approximation (the value of dielectric constant $\varepsilon = 1$), though the solvation effects in the framework of PCM model (Tomasi & Persico 1994) were taken into consideration for all forms of comenic acid with $\varepsilon = 10$ (which models the dielectric properties of the receptor binding pocket) and $\varepsilon = 78.3$ (aqueous solution).

Behavioral tests

Intracranial self-stimulation

Intracranial self-stimulation (ICSS) of brain areas forming the system of positive reinforcement is a widely applicable model for experimental evaluation of the positive emotional behavior in animals (Olds & Milner 1954; Zvartau 1977). This model allows studying effects of various pharmacological drugs on emotional behavior (Zvartau 1979). Intensification of ICSS behavior is a common property of medicines evoking drug dependence. The aim of this part of experiments was to investigate effects of comenic acid on functional activity of the lateral hypothalamic reinforcing system that is estimated as intensity of ICSS characterized by a lever pressings rate (Zvartau 1977). The lateral hypothalamus is considered the most active brain reward site (Gallistel 1985). Adult male *Wistar* rats (n=17, three-months old; 200–250 g), were obtained from the vivarium of I.P. Pavlov Institute of Physiology of the Russian Academy of Sciences. Rats were weighed, anesthetized intravenously with a combination of ketamin (3 mg/kg) and nembutal (30 mg/kg) and after treatment of the head skin with a local anesthetic (0.2% solution of novocaine) placed in a stereotaxic device. The peri-

osteum was scraped from the skull, it was dried with 5% perhydrol solution and insections were made on the skull for the electrode fastening with a dental acrylic. Two small holes were stereotaxically drilled bilaterally in the skull for the further insertion of stimulating bipolar concentric electrodes into the lateral hypothalamus (*A*=1.5 mm, *L*=1.5 mm, *H*=8.5 mm, according to the stereotactic rat coordinates (Paxinos & Watson 1986). The electrode was made of a stainless steel tube (0.8×40 mm^2), through the channel of which an insulated nichrome wire, 0.2 mm in diameter, coated with Epoxylite for adhesion to the tube, was passed. The tip of the wire emerged 0.8–1.0 mm beyond the tube. The entire electrode was coated with an insulating compound. The tips of both parts of the electrode were cleaned of insulation for 0.6–0.8 mm. The use of such an electrode makes it possible to achieve a very focal stimulation effect on the tip of the electrode. Five–seven days after the operation each rat was placed in an observation box (40×40×60 cm^3) with a lever which, when pressed, closed the circuit of the current. In this case, a burst of impulses for 0.5 s (impulse duration 0.5 ms at 100 Hz) was delivered from an ESU-2 stimulator to the electrode. The current (90–300 μA) was adjusted to produce the maximum rate of the lever pressing during ICSS behavior. The current intensity evoking a stable frequency of lever pressing was arbitrarily taken as the threshold. The lever pressing rate was recorded on a recording device during 10 min.

Three concentrations of comenic acid (1, 10 and 30 mg/kg, i.p.) were tested in experiments with ICSS. Comenic acid was diluted in 1.5 ml of Hank's solution (pH=7.4). The control rats (n=3) were injected with Hank's solution of the same volume. The customized computer program made it possible to construct a histogram of the number of lever-pressings during 10 min before the drug injection, immediately after injection, as well as 30 and 60 min later, with simultaneous treatment of the average value. Changes in the threshold current for ICSS were observed.

After the tests, the rats were sacrificed by an overdose of urethane and coagulation of ICSS sites was performed (2 mA, DC, 10 s). The brains were placed in 10% formalin solution. The positions of stimulating sites were determined histologically in frozen sections (60 μm thick) using the stereotactic atlas (Paxinos & Watson 1986). Histological analysis indicated that the stimulating electrodes were localized in the lateral hypothalamus.

Formalin test

Formalin test (Dubuisson & Dennis 1977) is widely used for evaluation of tonic inflammatory pain and analgesic effects of various pharmacological drugs (Aloisi *et al* 1995; Wheeler-Aceto & Cowan 1991; Shields *et al* 2010; Barrot 2012). The formalin test we used as previously described (Butkevich *et al* 2009). Adult male *Wistar* rats (n=14, three months old; 240–280 g), obtained from the vivarium of I.P. Pavlov Institute of Physiology of the Russian Academy of Sciences, were used in the study. Experiments were carried out between 10:00 and 14:00 h. The experimental rats (n=7) were injected with comenic acid diluted in Hank's solution (1 ml, 30 mg/kg, i.p.); control rats (n=7), with Hank's solution of the same volume. Five minutes after drug administration, the animals were exposed to a subcutaneous formalin injection (2.5%, 50 μl) into the pad of the left hind paw. Immediately after injection each rat was placed into the experimental chamber (25×25×25 cm) with transparent walls, and the number of flexes+shakes and licking duration were recorded during an hour using a customized software, which makes it possible to record, quantify and analyze the pain-related behavior. Flexes and shakes patterns are the typical expression of inflammatory pain-related behavior in the formalin test (Wheeler-Aceto & Cowan 1991). This test allows to evaluate both acute nociception (the first phase, 5–10 min after formalin injection) and tonic persistent nociception (the second phase, duration about 30–40 min). Specific pain-related flexes and shakes patterns in response to formalin are organized at the spinal level; licking behavior, at the supraspinal level.

Hot-plate test

Analgesic action of comenic acid was evaluated in mice by the hot-plate test. In all hot-plate experiments referred to hereafter, comenic acid was applied in the form of 1% solution in sterile water for injections with 0.55% $NaHCO_3$ added. This solution is the finished medicinal product of a novel non-opioid analgesic Anoceptin comprising comenic acid as an active substance.

White mice (n=20, 2–2.5 months old; 18–20 g) were obtained from Rappolovo vivarium (Leningrad district) and acclimatized for 14 days. The temperature of the experimental plate was 52 °C. Pain thresholds were determined by measuring the latency to nociceptive responses. The latency was considered as the time of appearance of hind paw shake or lift, or jumping activity and recorded manually with a chronometer. The measurements were carried out 30 min, 60 min and 120 min after comenic acid injection. Comenic acid (Anoceptin) and Metamizole (Analginum) as a drug comparison were administered intravenously into the caudal vein with an insulinic syringe.

Statistical analysis

The data were processed with Student's *t* test. A *p*-value of ≤0.05 was considered to be statistically significant.

Results

Patch-clamp data

Rat DRG nociceptive neuron membrane was investigated using the whole-cell patch-clamp method. Extracellular application of comenic acid results in a decrease

of the effective charge transfer (Z_{eff}) in $Na_V1.8$ channel activation gating system. The limiting-slope procedure (Almers 1978) is used in the current study to estimate Z_{eff}. The approach has been described in the prior publications (Lopatina *et al* 2012; Yachnev *et al* 2012; Katina *et al* 2013). 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. $G_{Na}(E)$ could be obtained in the patch-clamp experiments as:

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

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

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

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 ($E\to -\infty$). The slope of the asymptote passing through the first points determined by the most negative values of E makes it possible to estimate the Z_{eff} value (Equation 1). It can be done so because at these potentials the Boltzmann's principle is applicable, where k is the Boltzmann constant, T is the absolute temperature, C is a constant, e_0 is the electron charge.

The families of $Na_V1.8$ channel currents in the control experiment and after extracellular application of comenic acid are presented in Figure 2. The corresponding peak current-voltage curve shifts slightly to the right after comenic acid application (Figure 2). The left branch of this function is more flat than in the control experiments. The voltage dependencies of the chord conductance are also different at negative E between the control and comenic acid data (Figure 3, A). When the chord conductance dependencies are obtained, the Almers limiting-slope procedure (Equation 1) can be applied, making it possible to evaluate Z_{eff} at the most negative potentials E (Figure 3, B). A very pronounced decrease in Z_{eff} after extracellular application of comenic acid or MPCA occurs due to activation of a receptor-coupled membrane mechanism (Figure 4). Indeed, a non-specific opioid antagonist naltrexone (NTX) switched off the effect of both acids (Figure 4). Z_{eff} also did not significantly change after a combined application of comenic acid (or MPCA) and ouabain, a specific blocker of Na^+,K^+-ATPase, at 200 μM (Figure 4). Ouabain applied at this rather high concentration totally inhibits both the pumping and transducing functions of Na^+,K^+-ATPase, therefore interrupting the signal transduction cascade initiated by binding of gamma-pyrones to the opioid-like receptor (Krylov *et al* 2000; Lopatina *et al* 2012).

Thus, according to our findings, $Na_V1.8$ channels in nociceptive neuron membrane should be under effective control of opioid-like receptors and transducing (non-pumping) function of Na^+,K^+-ATPase (Krylov *et al* 2000; Xie 2001; Yachnev *et al* 2012). In other words, comenic acid and MPCA do not interact directly with $Na_V1.8$ channel by the "modulated receptor" mechanism (Hille 2001). Our results strongly indicate that

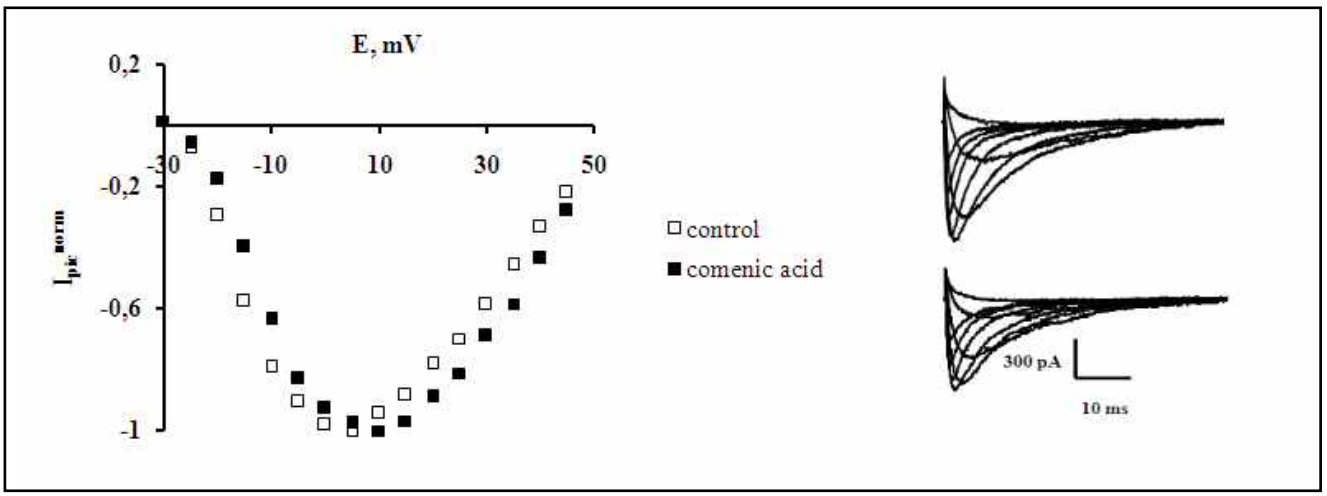

Fig. 2. Normalized values of peak current-voltage curves of $Na_V1.8$ channels. The functions are plotted from the results of the control experiments and after application of comenic acid at 100 nM. Inset: families of TTX-resistant sodium currents before and after application of comenic acid at 100 nM. The test potential was changed from –30 mV to 40 mV with a step of 10 mV. The holding potential of 500-ms duration was equal to –110 mV in all records. The leakage and capacitive currents were subtracted automatically.

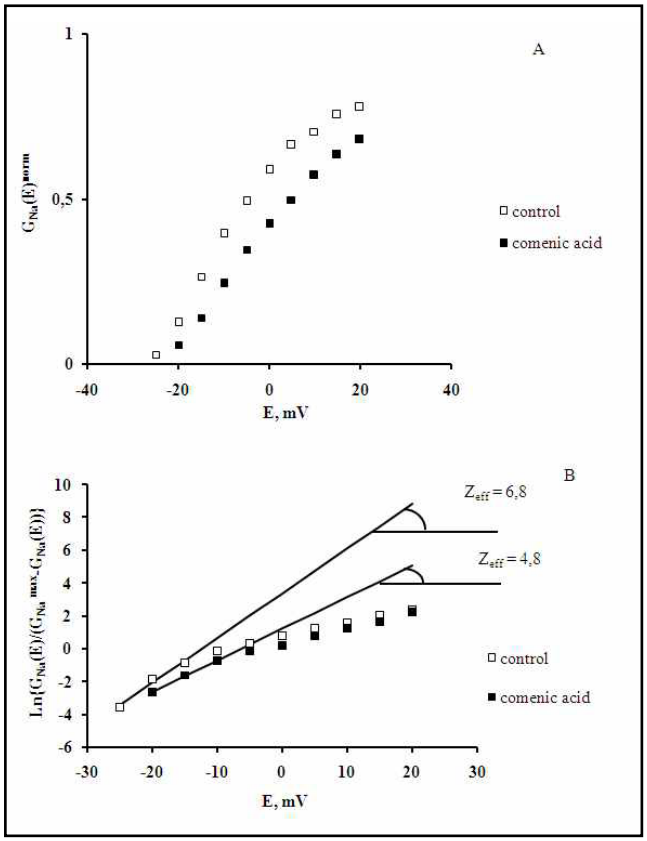

Fig. 3. Effect of comenic acid on the voltage sensitivity of the activation gating system of $Na_V1.8$ channels. **A.** Voltage dependence of the chord conductance of $Na_V1.8$ channels. The function $G_{Na}(E)$ was normalized, i.e., we plotted $G_{Na}(E)^{norm} = G_{Na}(E)/ G_{Na}^{max}(E)$, where $G_{Na}^{max}(E)$ is the maximal value of $G_{Na}(E)$. The results of the control experiments and after application of comenic acid at 100 nM are presented. **B.** Evaluation of the effective charge transfer of the activation gating system of $Na_V1.8$ channels. The exponential function presented in the logarithmic scale (the axis of ordinates) makes it possible to evaluate Z_{eff} from the slopes of the asymptotes passing through the first points determined by the most negative values of the membrane potential in the control experiments and after application of comenic acid at 100 nM.

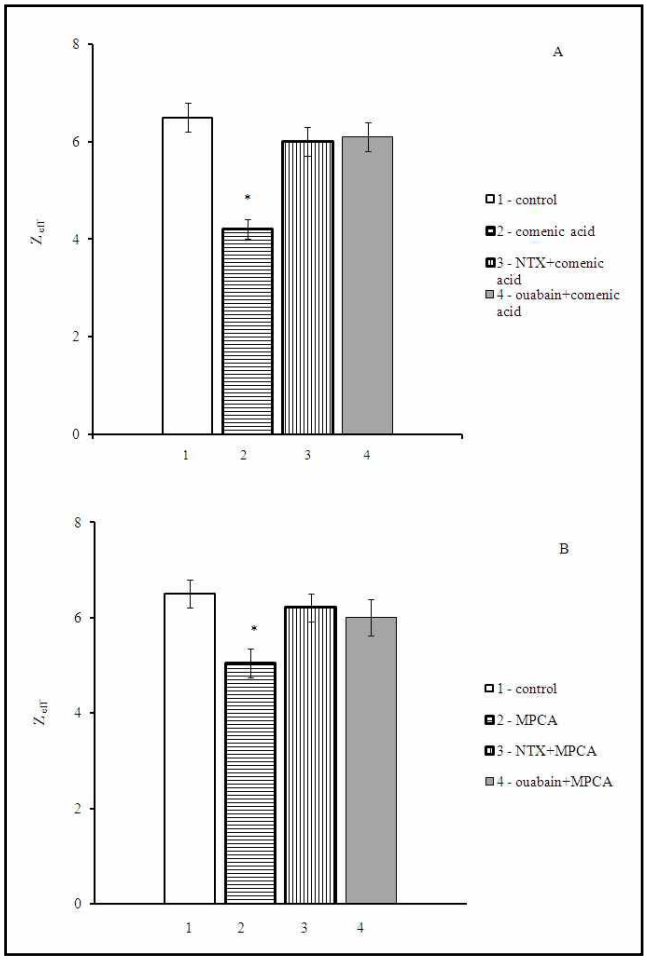

Fig. 4. Naltrexone and ouabain block the decrease of $Na_V1.8$ channel voltage sensitivity resulting from application of comenic acid (A) and MPCA (B). **A.** The control value of Z_{eff} = 6.5 ± 0.3 (n=22). Z_{eff} reduced to 4.2 ± 0.2 (n=18) after application of comenic acid at 1 μM. A combined application of naltrexone (NTX) and comenic acid at 50 μM did not result in a decrease of the effective charge (6.0 ± 0.3, n=12), as well as a combined application of ouabain (200 μM) and comenic acid (6.1± 0.3, n=15). * – difference between experimental and control data is statistically significant
B. The control value of Z_{eff} = 6.5 ± 0.3 (n=22). Z_{eff} reduced to 5.1 ± 0.3 (n=20) after application of MPCA at 100 nM. A combined application of naltrexone (NTX) at 50 μM and MPCA did not result in a decrease of the effective charge (6.2 ± 0.3, n=18), as well as a combined application of ouabain (200 μM) and comenic acid (6.0 ± 0.4, n=17). * – difference between experimental and control data is statistically significant.

these agents bind to an opioid-like membrane receptor, which is negatively coupled to activation gating devices of $Na_V1.8$ channels via intermediate membrane molecules that transduce the gamma-pyrones signal. Usually this role is played by G-proteins, whereas in the present case the signal transducer is Na^+,K^+-ATPase. This mechanism is in line with our prior data describing the nociceptive effects of low-power infrared irradiation (Lopatina *et al* 2012; Yachnev *et al* 2012).

It is extremely important that not all gamma-pyrone derivatives can decrease the voltage sensitivity of $Na_V1.8$ channels, though all molecules studied here share a very similar chemical structure (Figure 1). Comenic acid and MPCA pronouncedly change the Z_{eff} value, while chelidonic acid and kojic acid do not (Figure 5).

Quantum-chemical calculations

The receptor that binds comenic acid and MPCA is referred to as the opioid-like receptor primarily because a non-selective opioid antagonist NTX is demonstrated to block the action of gamma-pyrones. It is an yet structurally unidentified membrane receptor controlling the activation of an additional Na^+,K^+-ATPase-coupled signalization pathway that does not have much in common with the signaling cascades triggered by activation of opioid receptors described to date. Morphine was however shown to bind to this opioid-like receptor (Krylov *et al* 2000), whereas another very potent

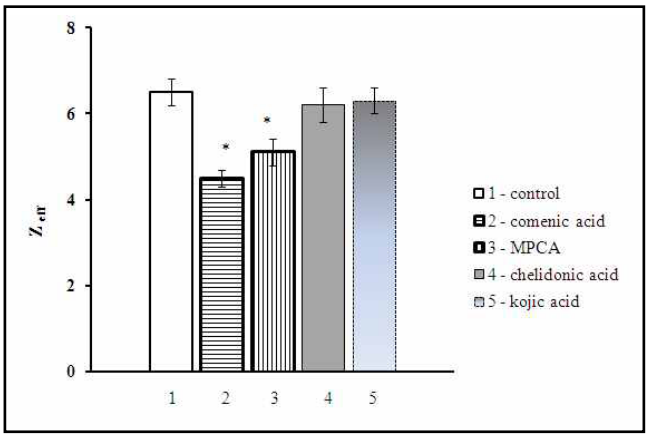

Fig. 5. Influence of gamma-pyrones on the effective charge of $Na_V1.8$ channel activation gating system. The control value of Z_{eff} = 6.5±0.3 (n=22). All gamma-pyrones were applied at 100 nM. Z_{eff} after application of comenic acid was equal to 4.5±0.2 (n=17); MPCA, 5.1±0.3 (n=20); chelidonic acid, 6.2±0.4 (n = 20); kojic acid, 6.1±0.3 (n = 21). * – difference between experimental and control data is statistically significant.

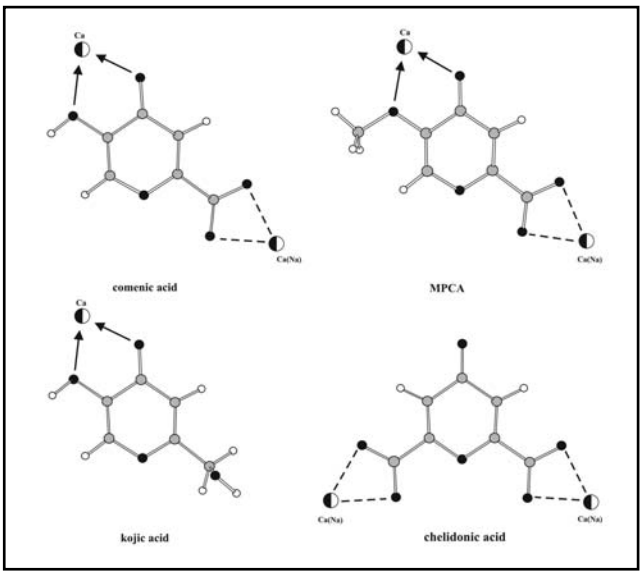

Fig. 6. RHF/6-31G* optimized structures of gamma-pyrones in a molecular form that involves the maximal number of bound cations. Black circles – oxygen atoms, gray circles – carbon atoms, white circles – hydrogen atoms, half-filled circles – cation atoms. Chelating bonds are presented with arrows; salt bonds, with dashed lines.

and selective μ-opioid agonist endomorphine did not exhibit any ability to activate it and thus control the effective charge transfer in $Na_V1.8$ channels (Katina *et al* 2003). Morphine belongs to a class of relatively small cationic ligands; hence the opioid-like receptor should share the structural features of other receptors for cationic ligands.

Small cationic ligands usually have at least one positively charged moiety that is generally thought to bind to an acidic residue of the receptor (Strader *et al* 1994). Two aspartate residues were found to play a crucial role in the ligand binding: one of them is absolutely conserved in most families of ligand-activated G-protein-coupled receptors, whereas the second one is absolutely conserved in adrenergic, muscarinic, and opioid receptors, as well as in other receptors for small cationic ligands (Zhorov & Ananthanarayanan 2000). It is highly probable that the opioid-like receptor also contains these residues. None of the compounds in the present study possesses positively charged groups, which led to the assertion that gamma-pyrones bind to the opioid-like receptor with assistance of one or several inorganic cations.

Carboxy groups in the α-position to the pyrone ring oxygen can form salts with inorganic cations, whereas the hydroxy and the carbonyl groups in adjacent positions of the pyrone ring are known to chelate divalent cations in water and 50% aqueous dioxane (Bryant & Fernelius 1954; Okáč & Kolařík1959; Petrola 1985). In patch-clamp experiments all agents were added to the extracellular solution containing Na^+, Ca^{2+} and Mg^{2+} at concentrations of 65 mM, 2 mM and 2 mM, respectively. Comenic and kojic acids were shown to form stable chelates with Ca^{2+} and Mg^{2+} in 1:1 stoichiometry, given that the cation concentrations were at least ten times higher than those of the chelating agents (Bryant & Fernelius 1954; Okáč & Kolařík 1959). Monovalent cations at high concentrations did not substantially affect the stability constants of chelate complexes of these gamma-pyrones with Ca^{2+} and Mg^{2+}. The electron-accepting ability of Ca^{2+} is higher than that of Mg^{2+}, and both cations are present in the extracellular solution at equal concentrations. This leads to the assumption that Ca^{2+} is the cation which forms the complex with the chelating moiety of the ligands under consideration.

To investigate the involvement of inorganic cations in the ligand-receptor binding process, a full geometry optimization of comenic acid, chelidonic acid, kojic acid and MPCA in various molecular forms was performed. The optimized structures of comenic acid, MPCA, kojic acid and chelidonic acid in a molecular form that involves the maximal number of bound cations are presented in Figure 6. The results of the calculations demonstrate that the spatial and electronic structures of these molecules are quite similar. The minor differences detected are attributed to the variations in origin of the pyrone ring substituents and in the intramolecular hydrogen bonding patterns. The PCM calculations do not indicate any substantial structural changes upon solvation of comenic acid and its derivatives. The processes of Ca^{2+} chelation and formation of Ca^{2+} and Na^+ salts are energetically allowed, and no steric restraints for Ca^{2+} chelation are found. As the molecules in study share a very similar chemical structure, it can be concluded that the inability of chelidonic and kojic acids to bind to the opioid-like receptor, and therefore the lack of the physiological effect of these substances, is due to their inability to interact with two inorganic cat-

ions simultaneously. Chelidonic acid lacks the hydroxy group and cannot chelate Ca^{2+}, whereas kojic acid lacks the carboxy group and cannot forms salts at physiological pH.

We suggest that the active gamma-pyrones (comenic acid and MPCA) bind to the opioid-like receptor as the salt of Ca^{2+} chelate, interacting with conserved aspartates of the receptor during ligand binding. The chelated Ca^{2+} forms a salt bridge with one of the aspartates, while the other aspartate interacts with the cation which serves as a counterion for the carboxy group of the ligand.

To provide the complementarity between the gamma-pyrone molecules and the opioid-like receptor binding pocket, the distance between pyrone-bound cations should match with the distance between the aspartates in the receptor. The intercationic distances in the salts of Ca^{2+} chelates of comenic acid and MPCA were calculated to be between 0.94 nm and 0.96 nm, and did not depend significantly on the molecular structure of ligands, origin of the cation (Na^+ or Ca^{2+}) forming salts with the carboxy group and the dielectric constant of the milieu. The molecules of comenic acid and MPCA are very rigid; therefore this distance may be regarded as a structural determinant which defines the distance between two active electrophilic centers in ligands of the opioid-like receptor. The side chains of the conserved aspartates are more flexible, making it possible to adjust the spatial orientation of acidic functional groups of the receptor in the process of formation of intermolecular ion-ionic bonds essential for ligand-receptor binding.

The results of quantum-chemical calculations thus provide an explanation for experimentally obtained in patch-clamp investigations data regarding the receptor-coupled modulation of $Na_V1.8$ channels by gamma-pyrones. To further clarify the physiological role of this molecular mechanism, the behavioral tests were performed.

Behavioral tests

Intracranial self-stimulation

The number of lever pressings in experimental rats was from 220 to 550 per 10 min. The threshold current value for stable lever pressing rate ranged from 90 to 330 μA. Injection of the control solution did not change the lever pressing rate. The average number of lever pressings per 10 min before injection of comenic acid was 295±13, which was taken as 100%.

Injection of comenic acid at 1 mg/kg (n=3) failed to evoke any changes in both lever pressing rate and the threshold current value immediately after injection and in the following periods of observation (30 and 60 min after injection). The control rats (n=3) injected with Hank's solution of the same volume also did not show any changes in indices under study. Just after injection of comenic acid at 10 mg/kg (n=3) the lever pressing rate increased slightly, but 30 and 60 min later this index and the threshold current value decreased significantly ($p<0.05$). Injection of comenic acid at 30 mg/kg (n=8) induced an immediate significant decrease ($p<0.01$) in ICSS as compared with the control, which retained 30 and 60 min after injection (Figure 7).

The results of the present study demonstrate that comenic acid induces a decrease in ICSS of the lateral hypothalamus. Application of comenic acid at 30 mg/kg results in the most pronounced decrease in the lever pressing rate. Thus, injection of comenic acid compensates for the reward-dependent effect evoked by the action of the stimulating current on the positive rewarding system of the lateral hypothalamus. The data obtained strongly indicate that injections of comenic acid do not produce any drug dependence and euphoria. It is tempting to suggest that comenic acid also will not produce these negative effects in humans.

Formalin test

The purpose of the current experiments was to determine effects of comenic acid on the behavioral indices of inflammatory pain in the formalin test in adult male rats. Injection of comenic acid (30 mg/kg, i.p.) before the formalin test reduced the licking duration in the first acute phase (10.3±3.8 and 37.3±5.6 s, p=0.003) and in the second tonic phase (76.1±17.2 and 265.0±37.0 s, $p\leq0.0001$) as compared with the control, and also decreased the number of flexes+shakes in the first (25.7±7.3 and 61.1±10.7, p=0.019) and the second (154.0±40.5 and 607.6±97.5, p=0.001) phases as compared with the control (Figure 8).

It can be concluded that an analgesic effect of comenic acid was detected in the acute and tonic phases in the formalin test. This drug substance alleviated pain at both the spinal and supraspinal levels.

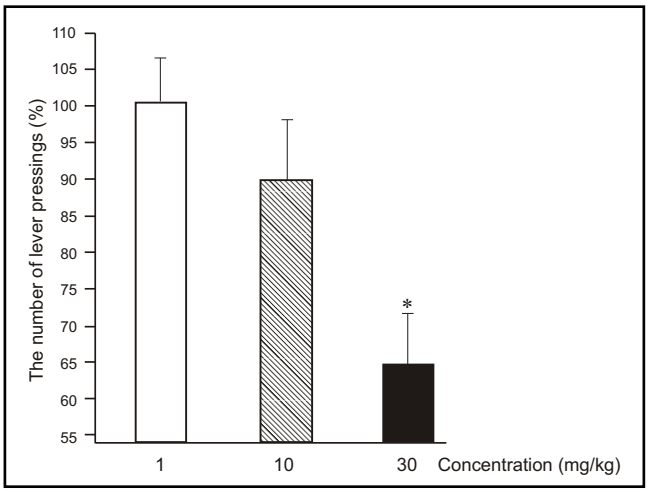

Fig. 7. Intensity of ICSS depending on the concentration of comenic acid. The axis of abscisses, comenic acid concentration (mg/kg). The axis of ordinates, the number of lever pressings (in % to the average number of lever pressings before injection of comenic acid taken as 100%). * p<0.01.

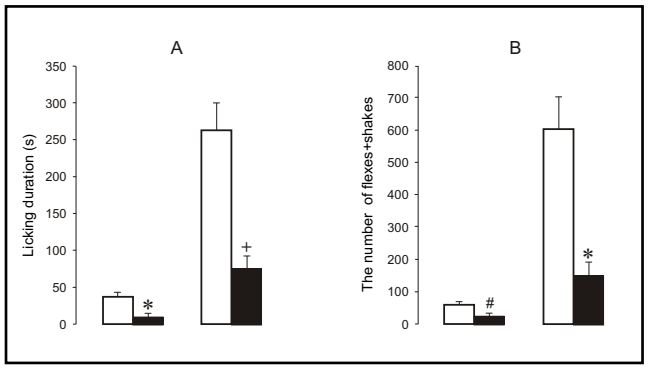

Fig. 8. Effects of comenic acid on licking duration and the number of flexes+shakes in the formalin test in rats. Licking duration (**A**), the number of flexes+shakes (**B**) in the formalin test. White columns – controls, black columns – experimental rats after comenic acid administration. Data are means±S.E.M. #p<0.05, *p<0.001, +p<0.001 as compared with appropriate controls.

Tab. 1. Influence of comenic acid and Metamizole on pain sensitivity in mice in the hot-plate test, M±m.

Intravenous injection	Latency (s) after injection		
	30 min	60 min	120 min
Control	10.5±0.9	10.8±0.6	11.1±0.8
Anoceptin (comenic acid, 5 mg/kg)	22.0±1.1*	21.7±0.7*	22.3±0.8*
Anoceptin (comenic acid, 10 mg/kg)	31.8±0.7*	32.5±1.4*	33.7±1.2*
Anoceptin (comenic acid, 50 mg/kg)	37.0±1.6*	37.2±1.9*	38.2±1.8*
Analginum (Metamizole, 50 mg/kg)	20.1±0.6*	22.1±1.8*	22.5±0.9*

*– difference between experimental and control data is statistically significant, p<0.05

Hot-plate test

Comenic acid was administered as Anoceptin, which is the finished medicinal product of a novel non-opioid analgesic in the form of 1% comenic acid solution in sterile water for injections with 0.55% $NaHCO_3$ added. Intravenously injected comenic acid exhibited a pronounced dose-dependent analgesic action in the concentration range from 5 to 50 mg/kg of the active substance (Table 1). The average effective concentration of comenic acid was 25 mg/kg. The latency of appearance of motor anxiety and jumping activity after administration of comenic acid at 5 mg/kg did not differ significantly from that after injection of Metamizole (Analginum) as a drug comparison at 50 mg/kg. Analgesic effect of comenic acid at 10 mg/kg and 50 mg/kg was significantly stronger than that of Metamizole at 50 mg/kg and was persistent for more than 2 hours after comenic acid injection at the conditions of the hot-plate test. Solution of 0.55 % $NaHCO_3$ in sterile water for injections was used as the control (Table 1).

Discussion and Conclusions

To create a new analgesic we use an approach based on receptor-coupled activation of membrane signaling pathway, which involves $Na_V1.8$ channels (Krylov *et al* 2000). It is an inhibiting mechanism that is responsible for strong analgesic effect of comenic acid. Morphine was also found to decrease $Na_V1.8$ channel voltage sensitivity (Krylov *et al* 2000). As the non-specific opioid antagonist NTX blocked the effect, it was suggested that morphine interacted with an yet structurally unidentified opioid-like receptor, thus triggering the further transduction of the signal to $Na_V1.8$ channels. G-peptides were shown not to be involved in this mechanism: the application of GTPgammaS, GTPbetaS and choleric toxin did not influence the voltage sensitivity of $Na_V1.8$ channels. The role of the signal transducer in this machinery is played by Na^+,K^+-ATPase (Krylov *et al* 2000). This novel transducing function of Na^+,K^+-ATPase was detected not exclusively in the neuronal membrane (Yachnev *et al* 20012; Lopatina *et al* 2012), but also in cardiomyocyte cells (Xie 2001). Comenic acid can effectively substitute morphine as an activator of this signaling mechanism. Moreover, it activates only opioid-like receptors coupled to $Na_V1.8$ channels, but not opioid receptors.

Comenic acid (as a salt of Ca^{2+} chelate complex) interacts with the membrane opioid-like receptor coupled to $Na_V1.8$ channels by forming ion-ionic ligand-receptor bonds. It is a key step in the ligand binding. The distance between the cations bound by a comenic acid molecule is calculated to be approximately 1 nm, and it should match with the distance between two negatively charged functional groups in the opioid-like receptor locus that controls the ligand recognition process. Our patch-clamp data and quantum-chemical calculations provide an explanation for the observed physiological effect of gamma-pyrones using this very simple idea. Only those gamma-pyrones, which bind at least two cations at the appropriate intercationic distance, can modulate the voltage sensitivity of $Na_V1.8$ channels (Figures 5–6). Comenic acid perfectly fits the requirements and is therefore chosen for the further analysis and development of the injection form of an analgesic medicine that contains comenic acid as an active substance. Results of behavioral tests unequivocally indicate that comenic acid applied at milligram per kg concentrations exhibits a very pronounced analgesic effect. The data obtained *in vivo* strongly supports the suggestion that $Na_V1.8$ channels are the key molecules that control the pain sensation. A decrease in excitability of primary and secondary neuron membranes due to receptor-coupled action of comenic acid results in pain relief at both spinal and supraspinal levels, as shown with the hot-plate and formalin tests.

The application of comenic acid does not promote the negative side effects, which is demonstrated with our experiments on positive reward zone stimulation

(Figure 7). Comenic acid administered at its "analgesic" milligram per kg concentrations switches on a very powerful physiological mechanism that effectively competes with the strong brain-formed lateral hypothalamus mechanism of electric stimulus-dependence.

The idea behind the present investigation is to substitute morphine for a more friendly and benign compound (comenic acid), which is capable to achieve chronic pain relief during its long-term application without negative side effects. Results of behavioral experiments support this prediction. They indicate that comenic acid does not switch on the opioid-controlled signaling cascades. It activates a novel additional nociceptive opioid-like-receptor-coupled mechanism that functions in parallel to well-known opioidergic system. The data obtained makes it possible to predict that new analgesic Anoceptin should be very effective and safe for humans. It also should not evoke the physical dependency and euphoria associated with action of morphine, other opiate alkaloids and their derivatives. The latter suggestions were successfully verified during the first phase of clinical trials. Report about clinical study of safety and pharmacokinetics was presented into the Scientific Center of expertise of tools of medical use of the Russian Health Supervision of the Russian Federation and was approved (protocol N 12 of June 05, 2009). Further clinical trials scheduling is under way.

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