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Frontiers in Pain Science

Volume 1

New Non-opioid Analgesics: Understanding Molecular Mechanisms on the Basis of Patch-clamp and Quantum-chemical Studies

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(Volume 1)

(New Non-opioid Analgesics: Understanding Molecular Mechanisms on the Basis of Patch-clamp and Quantum-chemical Studies)

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CONTENTS

PREFACE

In 1897, at a meeting of the Society of Russian Physicians, Ivan Pavlov predicted that the last stage of the life sciences would be the physiology of the living molecule. Nowadays the last stages of molecular approaches are theoretical quantum-chemical calculational techniques and experimental patch-clamp method which really can describe the behavior of living molecules. An attempt of combined application of quantum-chemical calculations and the patch-clamp method to investigation of the nociceptive system is presented in this volume. The crosstalk between drug substances and membrane receptors is conducted in the language of molecules. The behavior of single molecules upon their ligand-receptor binding should be investigated at physiologically adequate conditions during development of new analgesics. The requirement of physiological adequacy was always taken into account when the authors tried to explain the background mechanisms governing the effects of powerful analgesics. This approach makes it possible to elucidate how the chemical structure of labile attacking molecules should be finely tuned to provide effective binding to their membrane receptor. The authors hope that this review will open a new perspective to application of molecular methods in the drug design of pain relievers. The urgent need for the development of novel analgesics is dictated by the lack of safe and effective drugs in this field of medicine, especially when the pain becomes intolerable and incurable. The arsenal of practical medicine includes an array of analgesics, which have to be applied basing on the severity of pathological conditions of the organism. Step 1 of the World Health Organization analgesic ladder consists of non-opioids, administered with or without adjuvants depending on the type of pain. Step 2 comprises step 1 agents plus opioids which can relieve mild to moderate pain. Step 3 involves step 2 agents with addition of opioids for moderate to severe pain relief. It is a matter of common knowledge that administration of opioid substances results in irreversible adverse side effects in humans. The major objective of the authors is to solve this underlying problem by creating novel analgesics which could replace opioids in clinical practice, while remaining completely safe.

This book presents our main result in elucidation of the physiological role of a novel membrane signaling pathway involving the opioid-like receptor coupled to slow sodium channels ($Na_v1.8$) via Na^+,K^+-ATPase as the signal transducer. This 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 further triggering the signaling pathway directed towards $Na_v1.8$ channels provides the effectiveness and safety of our novel analgesic which is potent enough to relieve severe pain otherwise relieved exclusively by Step 3 opioids.

It is nowadays almost inevitable for reviewers of scientific material in the field of nociception to make excuses for omissions. We are sincerely sorry for not having been able to discuss all the findings in physiology of nociception and in practical medicine that would have merited attention. To include all would have defeated the purpose of this volume by making it grow out of all proportions.

This book presents an informative and valuable for physiologists and clinicians overview of primary molecular mechanisms involved in functioning of the peripheral nociceptive system. This material can be used in courses given to students specializing in physiology, psychology, and medicine, as well as to physicians training in neurology, neurosurgery, and psychiatry. The principles presented in the current volume may also be of interest to molecular biologists engaged in the drug design.

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CHAPTER 1

Introduction and Methodology

Abstract: Discovery of $Na_V1.8$ channels has opened a new perspective to study the mechanisms of nociception. A remarkable feature of these channels is their ability to be modulated by binding of various endogenous and exogenous agents to membrane receptors coupled to $Na_V1.8$ channels. The behavior of their activation gating system was patch-clamp recorded and analyzed by the Almers' limiting slope method. It was established that opioid-like membrane receptors could control the functioning of $Na_V1.8$ channels. A novel role in this mechanism is played by Na^+,K^+-ATPase, which serves as the signal transducer instead of G proteins. Switching on the opioid-like receptors one can selectively decrease the effective charge of $Na_V1.8$ channel activation gating device. As a result, only the high-frequency component of nociceptive membrane impulse firing is inhibited. This is the component that transfers nociceptive information to CNS.

The three units involved in the described membrane signaling cascade (opioid-like receptor → Na^+,K^+-ATPase → $Na_V1.8$ channel) 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.

Keywords: Impulse firing, Limiting slope procedure, $Na_V1.8$ channels, Na^+,K^+-ATPase, Nociception, Opioid-like receptor, Patch-clamp method.

PHYSIOLOGY OF PRIMARY SENSORY CODING

The universal language of the brain is the language of nerve impulses. In the 1920s Edgar Adrian was the first who discovered that discharge frequency of an afferent fiber innervating feline mechanoreceptors increased as a consequence of an increase in the stimulus intensity. The input-output function of the primary afferent fiber describes the relationship between the stimulus intensity and the number and frequency of evoked action potentials [1]. Different forms of energy are transformed by the nervous system into different sensations of sensory modalities. Five major sensory modalities have been recognized since ancient times: vision, hearing, touch, taste, and smell. In 1844 Johannes Müller advanced his "laws of specific sense energies" [2]. He proposed that modality was a property of the sensory nerve fiber. Each fiber is activated by a certain type of stimulus because different stimuli activate different nerve fibers. In turn, the nerve

fibers make specific connections within the nervous system, and it is these specific connections that are responsible for specific sensations. A unique stimulus that activates a specific receptor and therefore a particular nerve fiber was called an adequate stimulus by Charles Sherrington [3]. In 1967 Vernon Mountcastle advanced the idea of the brain as a "linear operator" [4, 5]. It means that the input-output functions of sense organs should be congruent with psychophysical functions relating the magnitude of the stimulus to the sensation. For instance, the function of central pathways mediating simple sensory events in the somatosensory system is thought to conserve the presentation of a stimulus dictated by the peripheral sensory apparatus. Said differently, one could assign for each discriminable quality of sensation a specific set of nerve fibers whose excitation would express that one quality (modality) and no other. The alternative view stated that quality was a matter of the pattern or of the spatio-temporal distribution of excitation in a whole array of fibers. As a result, the "labeled line" theory was opposed to the alternative "pattern" theory (see review) [6].

The "sixth" sensory modality, *i.e.* pain, up to now attracts special attention of physiologists and clinicians. It is difficult to overestimate the significance of attempts to control the mechanisms of pain sensation in order to achieve practical results regarding chronic pain relief in humans. The first steps in this direction have been done by researchers who laid the foundations of nociception as one of the most important branches of sensory physiology.

Alfred Goldscheider (1920) [7] was the first to advance the idea that the pain was not modality-specific but rather evoked by an additional excitation of any sense organ.

Ivan Pavlov (1927) [8] showed how the brain could be trained, through repetition, to invoke certain reactions in certain circumstances. Pavlov distinguished between food stimulations which called out the reaction of salivation and electric current noxious stimulations which called out the defense reaction. Destructive (noxious) stimuli provoke the defense reflex. Food calls for a positive reaction – grasping of the substance and eating it. Pavlov has shown that the defense reflex of skin is second in importance to the food reflex. An animal exposed simultaneously to an electric current acting upon his skin and to a food stimulus would respond not with defense but with food reaction. These findings show that mediation of nociceptive signals does not strictly obey the "labeled line" law. This "line" is under control of some other physiological processes of living organism.

Investigating the physiological nature of sleep Pavlov stated that sleep, or inhibition, prevented undue fatigue of the cortical elements, allowing them, after they had been subjected to noxious stimulation, to recover their normal state.

Inhibition is occurring all the time, even in a seemingly alert animal, but it exists only in scattered areas of the cortex. When it irradiates from these areas over the entire brain, the animal falls asleep. In Pavlov's words, "internal inhibition in the alert state of the animal represents a regional distribution of sleep which is kept within bounds by antagonistic nervous process of excitation" (Pavlov, Conditioned reflexes, p. 253) [8]. Pavlov has demonstrated that the nature of the stimulus itself is less important than the inhibition associated with it. "As there is practically no stimulus of whatever strength that cannot, under certain conditions, become subjected to internal inhibition, so also there is none which cannot produce sleep" (Pavlov, Conditioned reflexes, p.252) [8]. He mentions an instance in which a powerful electric shock applied to the skin was used as a conditioned alimentary stimulus that totally relieved pain (see also [9]).

There are three main consequences of Pavlov's findings. The first one is that his results corroborate the "pattern" theory, because as it was mentioned above, the "noxious labeled line" could be easily disrupted by signals of other modalities in an alert organism. The second consequence is the suggestion that nociceptive signals can be controlled somewhere at spinal and/or supraspinal levels. And finally, nowadays we can predict that endogenous substances which should control pain sensation on the molecular level are expressed in human brain.

A starting point of any sensation is the reception of signals evoked by activation of specialized sensory receptors (including nociceptors) providing information to CNS. Nociceptors inform us mainly about harmful external and internal stimuli or about tissue injury. Pain is the perception of an aversive or unpleasant sensation that originates from a damaged region of the body. Our "sixth sense" is a vitally important sensory experience that warns us on danger. Modern findings concerning the relationship between perception of pain and mechanisms of functioning of nociceptors show that any nociceptive perception involves an interconnection and elaboration of sensory inputs and pathways. Highly subjective and complicated nature of pain makes it difficult to diagnose and treat a number of chronic pain phenomena.

A noxious stimulus activates the nociceptor fiber by the fundamental mechanism of excitation of living cell. It is well known that nerve excitation evoked by mechanical stimulation results in production of gradual receptor current in primary receptors [10, 11] or generator current in secondary receptors [12] that elicits the single action potential or trains of nerve impulses. Insights into neural mechanisms for fine coding of tactile information in humans come from the works of Ake Vallbo and his colleagues who have systematically studied mechanoreceptors innervating the human hand skin. On the basis of information obtained on alert subjects they have proven that even single extra action potential

CHAPTER 2

Possible Mechanisms of Binding of Gamma-Pyrones to the Opioid-Like Receptor

Abstract: Derivatives of gamma-pyrone show their remarkable ability to trigger the novel mechanism of $Na_V1.8$ channels modulation described in Chapter **1**. Unlike morphine, which activates both opioid and opioid-like receptors, comenic acid specifically switches on the latter mechanism involving Na^+,K^+-ATPase as the signal transducer. It is extremely important that not any gamma-pyrone derivative can decrease the voltage sensitivity of $Na_V1.8$ channels, though all molecules studied herein share a rather similar chemical structure. A very productive approach which makes it possible to elucidate the peculiarities of ligand-receptor binding on the molecular level is combined application of quantum-chemical calculations and the patch-clamp method. Below we present our findings that explain a totally unevident result of highly selective binding of gamma-pyrone derivatives to the opioid-like receptor. Understanding of this mechanism opens up opportunities for creation of a novel class of analgesics.

Keywords: Ca^{2+} chelate complex, Gamma-pyrone derivatives, Limiting slope procedure, $Na_V1.8$ channels, Nociception, Opioid-like receptor, Patch-clamp method, Quantum-chemical calculations.

Pharmacological effects of gamma-pyrone derivatives, including radioprotective [1 - 3], antiviral [4], antidiabetic [5], and anticonvulsant [6] effects, were recently examined. Kojic acid was demonstrated to be able to protect human skin from pigmentation [7, 8]. Gamma-pyrones are regarded as potential anticancer drugs [9]. They also exhibit antileishmanial activity [10].

Kojic acid derivatives were found to effectively modulate histamine H3 receptors (H3R). The most affine compounds showed receptor binding in the low nanomolar concentration range [11]. The authors suggest that antagonists/inverse agonists of the H3R are able to increase the neurotransmitter content and may find their application in the therapy of cognitive diseases, sleep/wake disorders, epilepsy, obesity, pain, or allergic rhinitis. Several substances are progressing in clinical trials [11].

Boris V. Krylov, Ilia V. Rogachevskii, Tatiana N. Shelykh, Vera B. Plakhova

Fundamentally new opportunities for clinical use of gamma-pyrones were discovered by Alexey Shurygin who developed food additive Baliz-2 [12, 13]. Its main ingredients, comenic and meconic acids, exhibit a profound antibiotic, antibacterial, and regenerative activity. It is worth noting that Baliz-2 never expressed any negative side effects during its long history of clinical application in Russia. Our starting investigations of probable molecular mechanisms of meconic and comenic acid targeting were inspiring: the agents decreased voltage sensitivity of $Na_V1.8$ channels [14]. These findings opened a promising perspective for research of the role of gamma-pyrone derivatives in nociception (see also [15]).

PATCH-CLAMP INVESTIGATION OF GAMMA-PYRONES

The families of $Na_V1.8$ currents in the control experiment and after extracellular application of comenic acid (5-hydroxy-gamma-pyrone-2-carboxylic acid, substance A) are presented in Fig. (**2.1.a**). It is clearly seen that the amplitude values of the currents are decreased, which can find its partial explanation in the existence of "run-down" effect inherent to the patch-clamp method [16, 17]. However, the decrease of the channels density may also take place. The peak current-voltage curve shifts in the depolarizing direction after comenic acid has been applied (Fig. **2.1.b**). The left branch of the current-voltage function is steeper as a result of comenic acid application than in the control experiments. The voltage dependencies of normalized $G_{Na\_s}(E)$ functions also differ between the control and comenic acid data at negative E (Fig. **2.2.a**). When $G_{Na\_s}(E)$ dependencies are obtained, the Almers' limiting slope procedure can be applied, making it possible to evaluate Z_{eff} at the most negative potentials E (Fig. **2.2.b**). A very pronounced decrease in Z_{eff} after extracellular application of comenic acid occurs due to activation of the receptor-coupled membrane mechanism (Fig. **1.17**). Indeed, a nonspecific opioid antagonist naltrexone (NTX) switched off the effect of comenic acid (Fig. **2.3**). Z_{eff} also remained fairly unchanged after combined application of comenic acid and a specific blocker of Na^+,K^+-ATPase, ouabain, at 200 μM (Fig. **2.3**). Ouabain applied at this rather high concentration totally inhibits Na^+,K^+-ATPase, therefore interrupting transduction of the signal triggered by binding of comenic acid to the opioid-like receptor and sent to $Na_V1.8$ channels according to the mechanism proposed earlier [18]. Moreover, these findings indicate that comenic acid can be compared to morphine in its efficiency of $Na_V1.8$ channel modulation. It switches on the three background mechanisms: reduces the channels density, positively shifts $Na_V1.8$ channel activation gating process, and, most importantly, markedly decreases Z_{eff}. The latter process is of dose-dependent nature, showing opioid-like receptor binding in the nanomolar concentration range. The binding process is characterized by K_d = 100 nM and the Hill coefficient n = 0.5 [14].

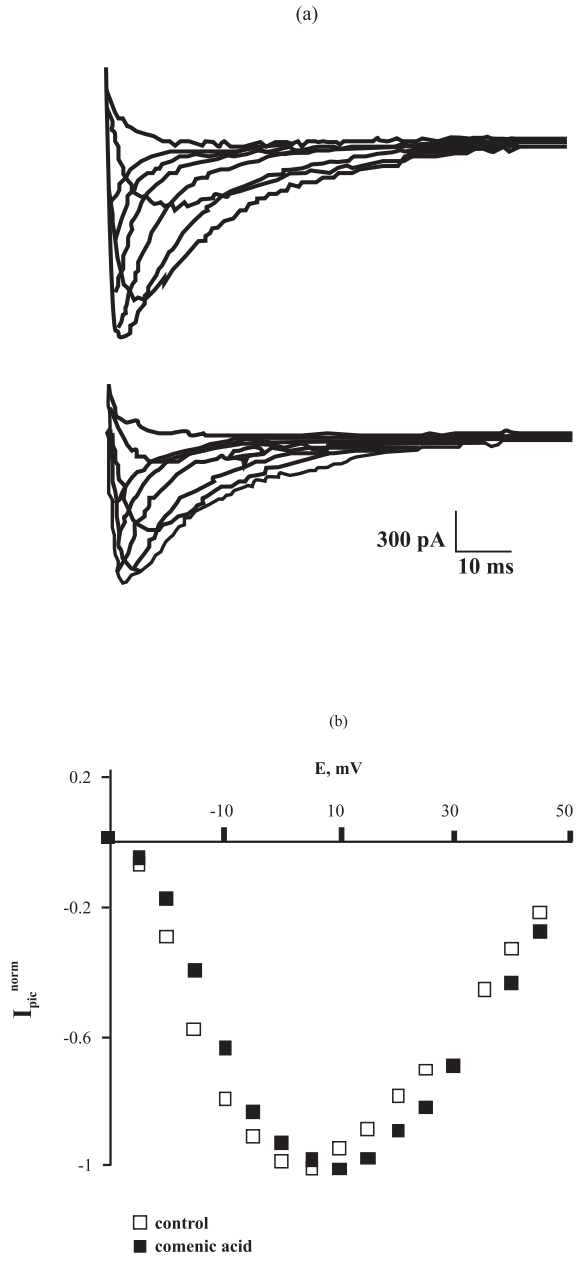

Fig. (2.1). Effects of comenic acid on $Na_V1.8$ channels.
a – Families of sodium currents measured in the control experiment (top) and after application of comenic acid at 100 nM (bottom);
b – Positive shift of the normalized peak current-voltage curve after application of comenic acid.

CHAPTER 3

Possible Mechanisms of Binding of Gamma-Pyridones to the Opioid-Like Receptor

Abstract: The nociceptive system codes noxious signals by increasing its impulse firing. $Na_V1.8$ channels play a central role in the process of primary sensory coding. The Almers' method is almost ideal for the study of behavior of their gating device. Application of this method makes it possible to elucidate the mechanism of receptor-coupled modulation of $Na_V1.8$ channels by opioid-like receptors, which exhibit high affinity to some gamma-pyrone and gamma-pyridone derivatives. A remarkable feature characterizing these substances is their ability to chelate calcium. That is why opioid-like receptors recognize these attacking molecules in physiologically appropriate conditions by activation of a very important additional mechanism of ion-ionic interactions switched on by attacking molecules with chelated calcium. This conclusion is confirmed by the study of the effects of gamma-pyridone derivatives, which are structurally very close to gamma-pyrones and, in addition, also have an ability to chelate calcium.

The results discussed in this and the previous chapters open a new approach to solve the problem of recognition of medicinal analgesic substances. Our quantum-chemical calculations demonstrate that calcium chelation process plays an important role in ligand-receptor binding and it is energetically allowed not only in vacuum but also in the adequate physiological environment. Conclusions concerning the probable structure of opioid-like receptor binding pocket are presented.

Keywords: Ca^{2+} chelate complex, Gamma-pyridone derivatives, Limiting slope procedure, $Na_V1.8$ channels, Nociception, Opioid-like receptor, Patch-clamp method, Quantum-chemical calculations.

The main result obtained in Chapter **2** of this book is elucidation of the physiological role of calcium chelation by gamma-pyrone derivatives, which is fundamentally important for ligand-receptor binding. Participation of the calcium ion in binding of gamma-pyrones to the opioid-like receptor is completely not obvious, and it allows introducing a new approach to analyze physiological activity of potential analgesics. This conclusion can be additionally verified by investigation of physiological effects of molecules relating to a class of substances, similar in structure to gamma-pyrones. We have chosen for our

further studies gamma-pyridone derivatives, which are distinguished from gamma-pyrones by the nature of the ring heteroatom (gamma-pyridones contain a nitrogen atom, as opposed to an oxygen atom in gamma-pyrones).

Many gamma-pyridones are demonstrated to be of pharmacological importance: they can exhibit analgesic activity [1 - 5], display anti-inflammatory [3, 4, 6], antitumor [7, 8], antibacterial [9, 10], antimicrobial [11], antimalarial effects [12], positively influence the cardiovascular system [13] and can also be applied for the treatment of Parkinson's disease [14].

3-Hydroxy-gamma-pyridone derivatives are widely used in various fields of medicinal chemistry to treat the diseases caused by excess microelements in serum [15 - 17], as well as to design radioactive and fluorescent labels for diagnostics [17]. These applications of 3-hydroxy-gamma-pyridones are based on their ability to chelate doubly and triply charged cations (Al^{3+}, Fe^{3+}, Ga^{3+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, Mg^{2+}) [17 - 20] through the carbonyl and hydroxyl groups at contiguous positions of the pyridone ring.

PATCH-CLAMP INVESTIGATION OF GAMMA-PYRIDONES

Insofar as gamma-pyridones are structurally related to gamma-pyrones discussed in Chapter **2**, it is of interest to investigate the effect of the ring heteroatom on physiological and structural properties of these compounds in order to find a new mechanism or new agents capable of acting as analgesics by selective modulation of $Na_V1.8$ channels. Two molecules were chosen to examine their ability to decrease the effective charge of $Na_V1.8$ channel activation gating device: 5-hydroxy-1-methyl-gamma-pyridone-2-carboxylic acid (substance E) and 5-hydroxy-2-hydroxymethyl-gamma-pyridone (substance F). Their structural formulae are presented in Fig. (**3.1**). It is important that the latter compound is a structural analog of kojic acid (substance D, Fig. **2.8**) which showed no appreciable activity in our patch-clamp experiments, while the former one is a structural analog of comenic acid (substance A, Fig. **2.8**) which is capable to activate the opioid-like receptor being applied at nanomolar concentrations.

The families of $Na_V1.8$ currents in the control experiment and after extracellular application of substance E are presented in Fig. (**3.2.a**). It is clearly seen that the amplitude values of the currents are reduced, indicating that the decrease of the channels density may take place. The peak current-voltage curve shifts in the depolarizing direction after substance E has been applied (Fig. **3.2.b**). The voltage dependencies of normalized $G_{Na\_s}(E)$ functions differ between the control and substance E data at negative potentials. In the latter case this function is steeper (Fig. **3.3.a**). When $G_{Na\_s}(E)$ dependencies are obtained, the Almers' limiting slope procedure can be applied, making it possible to evaluate Z_{eff} at the most negative

potentials (Fig. **3.3.b**). Our experimental results demonstrate that substance F is also able to decrease Z_{eff} of $Na_V1.8$ channel activation gating system. Its action is characterized by only two manifestations of inhibitory properties: it reduces the amplitude values of the currents (Fig. **3.4.a**) and decreases Z_{eff} (Fig. **3.5**). Somewhat unexpected in this case is the lack of the voltage shift of $Na_V1.8$ channels activation gating process (Fig. **3.4.b**). On the contrary, the effects of substance E are absolutely of the same character as those observed after morphine or comenic acid application (Figs. **1.13**, **2.2**). The decrease in Z_{eff} after extracellular application of substance E occurs due to activation of the receptor-coupled membrane mechanism (Fig. **1.17**). Indeed, this conclusion is based on the fact that nonspecific opioid antagonist naloxone (NLX) switched off the effect of substance E (Fig. **3.6**). Fig. (**3.7**) summarizes the effects of investigated gamma-pyridone derivatives. Both substances E and F decrease Z_{eff}, which makes it possible to predict their antinociceptive action on the organismal level.

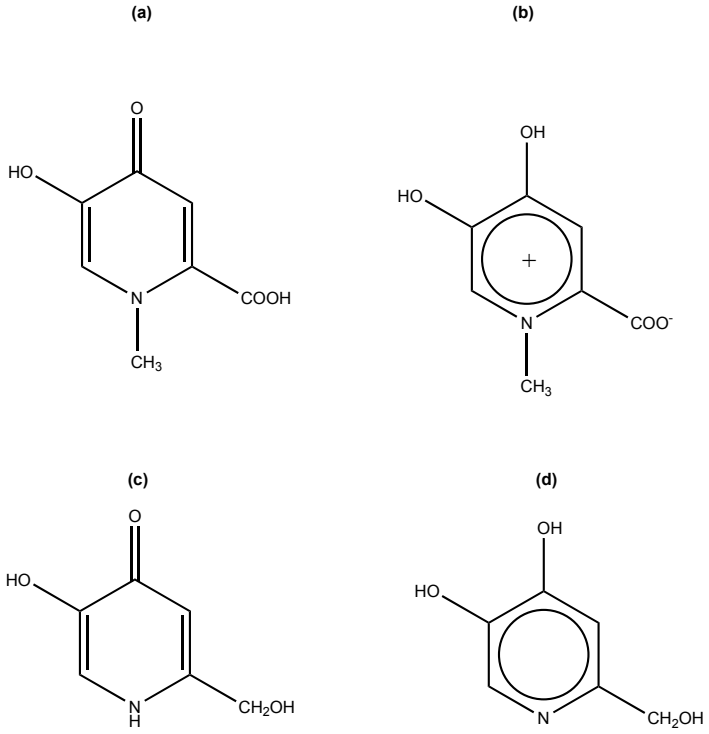

Fig. (3.1). Structural formulae of gamma-pyridones.
a – 5-hydroxy-1-methyl-gamma-pyridone-2-carboxylic acid (substance E), NH-form;
b – 5-hydroxy-1-methyl-gamma-pyridone-2-carboxylic acid (substance E), OH-form;
c – 5-hydroxy-2-hydroxymethyl-gamma-pyridone (substance F), NH-form;
d – 5-hydroxy-2-hydroxymethyl-gamma-pyridone (substance F), OH-form.

CHAPTER 4

Possible Mechanisms of Ligand-Receptor Binding of Cardiotonic Steroids

Abstract: Cardiotonic steroids are a recently discovered class of hormones synthesized in the adrenal cortex and hypothalamus and circulating in the blood. It is well known that the target molecule for these agents is Na^+,K^+-ATPase. A direct consequence of the proposed mechanism of $Na_V1.8$ channels modulation in Chapter **1** is the prediction of a special signaling function of the sodium pump. In other words, Na^+,K^+-ATPase should be involved in the processing of nociceptive information. The data presented in the current chapter support this idea. According to our findings, ouabain as a newly recognized hormone plays the role of endogenous analgesic at subnanomolar concentrations. Its target site is located directly on Na^+,K^+-ATPase and it recognizes ouabain, only in the form of its calcium chelate complex. The most significant result discussed in this chapter is explanation of the dual effect of ouabain: two distinct attacking molecules (ouabain and its calcium chelate complex) bind to two distinct sites of Na^+,K^+-ATPase, thus modulating two distinct functions of the enzyme: pumping and non-pumping (signal-transducing). Another newly recognized hormone, marinobufagenin, also exhibits analgesic effect at low concentrations but it is of principally different nature. This molecule lacks the ability to form marinobufagenin–Ca^{2+} chelate complex in 1:1 stoichiometry which could activate the signal-transducing function of Na^+,K^+-ATPase upon binding to the enzyme. The decrease of Z_{eff} of $Na_V1.8$ channel activation gating device induced by application of marinobufagenin at nanomolar concentrations results from activation of the "modulated receptor" mechanism, *i.e.*, this molecule binds directly to the aminoacid sequence of the channel without involvement of Ca^{2+}.

Keywords: Ca^{2+} chelate complex, Limiting slope procedure, Marinobufagenin, $Na_V1.8$ channels, Na^+,K^+-ATPase, Nociception, Ouabagenin, Ouabain, Patch-clamp method, Quantum-chemical calculations.

Endogenous cardiotonic steroids have various physiological functions. In particular, it was demonstrated that abnormal concentrations of these agents [1] could evoke different pathological states: congestive heart failure, cardiac arrhythmias [2, 3], hypertension [4], cancer [5], and depressive disorders [6, 7]. An increase of concentration of cardiotonic steroids was detected in the blood and subcutaneous water upon stress, lassitude, inflammatory processes in the

Boris V. Krylov, Ilia V. Rogachevskii, Tatiana N. Shelykh, Vera B. Plakhova

organism [8], pregnancy [9], and as a result of nephrectomy [10]. Endogenous cardiotonic steroids also influence cell growth and proliferation [11 - 13]. Ouabain exhibits anti-apoptosis action on endothelial cells [14]. Bufadienolides may induce apoptosis in human leukemia cells [15] and they also display antiproliferative activity and immunosuppressive activity upon action on T cells [16].

PATCH-CLAMP INVESTIGATION OF CARDIOTONIC STEROIDS

A direct consequence of our working hypothesis (Fig. **1.17**) is the prediction of a new mechanism of action of cardiotonic steroids, which should play an important role in nociception. When these agents activate the transducing function of Na^+,K^+-ATPase, they might produce an analgesic effect. In our opinion, the most interesting objects to study are endogenous substances, such as ouabain and marinobufagenin. Obviously, these agents can exert their analgesic properties only in vanishingly small "endogenous" concentrations, since it is known that high concentrations of cardiotonic steroids are extremely toxic. Endogenous ouabain was found in blood plasma in subnanomolar concentrations [17 - 19]. The designation "ouabain–Ca^{2+}" is used further to distinguish low (endogenous) concentrations of ouabain from its high concentrations, as in physiologically adequate conditions endogenous ouabain should exist in the form of calcium chelate complex (see below). Fig. (**4.1)** illustrates $Na_V1.8$ currents in the control experiment and after extracellular application of ouabain–Ca^{2+} at 1 nM. It is clearly seen that the amplitude values of the currents are decreased (Fig. **4.1.a**). The peak current-voltage curve shifts in the depolarizing direction (Fig. **4.1.b**) and the left branch of this function becomes steeper at negative E after ouabain–Ca^{2+} has been applied, which results in a very pronounced decrease in Z_{eff} (Fig. **4.2.a**) due to activation of the transducer-coupled membrane mechanism described in Chapter **1** (Fig. **1.17**). Indeed, a nonspecific opioid antagonist naltrexone (NTX) does not switch off the effect of ouabain–Ca^{2+} (Fig. **4.2.b**). These findings indicate that ouabain–Ca^{2+} can be compared to comenic acid or morphine in efficiency of $Na_V1.8$ channel modulation. It switches on the three background mechanisms discussed in Chapters **1-3** that should lead to pain relief: reduces the channels density, positively shifts $Na_V1.8$ channel activation gating process and, most importantly, markedly decreases Z_{eff}. The latter process is of dose-dependent nature, showing monotonic transducer-coupled ligand-receptor binding of ouabain–Ca^{2+} in subnanomolar and nanomolar concentration range from 100 pM to 10 nM (Fig. **4.3**). This binding process is characterized by K_d = **7** nM: extremely ouabain-sensitive branch of the dose-dependence curve reflects modulation of the signal-transducing function of Na^+,K^+-ATPase at the membrane level. As it was mentioned above, ouabain concentrations detected in human blood plasma are of the same order of magnitude (close to K_d). Thus, according to

our results, neuronal Na^+,K^+-ATPase as a signal transducer should be under effective control of endogenous ouabain. On the contrary, the right branch of Z_{eff} dependence on ouabain concentration is governed by a radically different background mechanism. An increase of ouabain concentration leads to inhibition of the pumping function of Na^+,K^+-ATPase. The second process can also be approximated by the Hill equation, the K_d value in this case being much higher (0.1 mM) (Fig. **4.3**). This fact leads to raising a very important question. How can one and the same enzyme (Na^+,K^+-ATPase) distinguish between its pumping and non-pumping functions modulated by one and the same molecule (ouabain)? The answer is partly given above: endogenous ouabain exists in the form of calcium chelate complex due to the presence of free calcium in small amounts in physiological medium. An increase of ouabain concentration, given that calcium concentration remains the same, results in an important effect: "free" ouabain binds to a completely different site of Na^+,K^+-ATPase. This fact will be explained below on the basis of our quantum-chemical calculations. Here it is worth noting that experimental evaluation of ouabain K_d carried out by different methods never led to unequivocal results, which could be in part accounted for by the heterogeneity of Na^+,K^+-ATPase isoforms. Four isoforms of its α subunit are known to exist, and they are expressed in a cell type-specific manner in higher vertebrates. Adult rat kidney and liver cells express the α1 isoform; glial and skeletal muscle, both α1 and α2; sperm cells, both α1 and α4. Unlike most other cells, neurons may express α1, α2, α3, or any combination of these isoforms. With rare exceptions, the α3 isoform of Na^+,K^+-ATPase is detected in neurons of adult vertebrates only [20]. Therefore it is not surprising that quantitative data concerning the mechanisms of binding of cardiotonic steroids to Na^+,K^+-ATPase scatter significantly. The K_d values describing these processes vary with isoform type and they are different in rodents and humans. Human α1, α2, α3, and α4 isoforms have K_d in the range of 10^{-9} to 10^{-8} M [21 - 23]. One of these isoforms (α1) is ouabain-insensitive in rodents and has a very high K_d value of about 10^{-6} M [24, 25]. Furthermore, binding of cardiotonic steroids to the α subunit of Na^+,K^+-ATPase is affected by the particular β subunit associated with it [26]. Taking into account the three α subunits (α4 appears to be present specifically in spermatozoa), the three β subunits and the seven FXYD subunits that have been shown to associate with Na^+,K^+-ATPase, there are potentially 63 different receptor complexes with which cardiotonic steroids can interact [27].

Our patch-clamp data indicate that there are at least two different ouabain binding sites in Na^+,K^+-ATPase (Fig. **4.3**). Investigations of ouabain-sensitive current of Na^+,K^+-ATPase in small neurons from adult rat dorsal root ganglia also demonstrated the existence of two ouabain binding sites, which were suggested to be located on two functionally distinct Na^+,K^+-ATPase isozymes, α1β1 and α3β1, with ouabain dissociation constants of 0.2 and 140.1 μM, respectively [28].

CHAPTER 5

Concluding Remarks

Abstract: Molecular mechanisms of the nociceptive information control in primary sensory neuron are described based on our investigation of the membrane signaling cascade (opioid-like receptor → Na^+,K^+-ATPase → $Na_V1.8$ channel). Summarizing the data presented in this volume it is possible to conclude that modulation of $Na_V1.8$ channels responsible for the coding of noxious signals can be carried out due to two novel targeting mechanisms. The first of these is the activation of opioid-like receptors; the second is the activation of the Na^+,K^+-ATPase signal-transducing function. Development of a novel class of analgesics that trigger these mechanisms should lead in the near future to successful solution of the problem of chronic pain relief.

Keywords: Analgesic, Modulated receptor, $Na_V1.8$ channels, Na^+,K^+-ATPase, nociception, Opioid-like receptor, Signal transducer.

It is known that pain is unpleasant but necessary. It signals of danger, preventing us from harming ourselves, and alerts on possible damage to our bodies. Too much pain is crippling and can make everyday living an agony. That is why pain and suicide are related. Even "good" pain can turn bad, when the pain caused by an injury persists after the damage has healed. Chronic pain dramatically reduces the quality of life for millions of people. There is no doubt that any steps to develop potent and safe analgesics are of major importance. Unfortunately, no analgesics in the arsenal of practical medicine satisfy these two criteria at the same time. However, there is always hope that other opportunities to fight pain are hidden within the human body. Even the smallest practical result in finding them is very important, because endogenous mechanisms of pain relief should have no negative side effects. In our opinion, to elucidate them it is necessary to link physiology, which is the basis of medical science, with calculational chemistry that makes it possible to describe physiological events on the detailed molecular level.

The basic physiological principles should be applied to analyze the fundamental mechanisms of nociception. Ivan Pavlov was the first who revealed a strong coupling between internal inhibition processes and antagonistic nervous process of excitation [1]. Intensity of sensory signals in the peripheral nervous system is

Boris V. Krylov, Ilia V. Rogachevskii, Tatiana N. Shelykh, Vera B. Plakhova

simply coded by frequency of nerve impulses. This principle discovered by Edgar Adrian [2] is widely used by us in this book. We also rely on the assumption of Vernon Mountcastle, who formulated it as the linear operator principle [3, 4]. We take this principle into consideration when we quantitatively describe the processes of receptor- or transducer-coupled modulation of $Na_V1.8$ channels. In other words, we postulate that the process of ligand-receptor binding that occurs in neighboring protein molecule linearly (or monotonically) influences the effective charge value of $Na_V1.8$ channels activation gating system.

We believe that the novel mechanism of $Na_V1.8$ channels modulation in nociceptive neuron (Fig. **1.7**) will open a new approach to solve the problem of chronic pain. Fig. (**1.7**) indicates the presence of three separate molecular targets. Each of them can interact with its "own" agonists and antagonists, some of which should be endogenous. It can be thus argued that the physiological effects of these interactions should result in the control of nociceptive signals. In accordance with our approach, antinociceptive response of sensory neuron can be obtained through activation of three different molecular mechanisms triggered by three different targets: opioid-like receptor, Na^+,K^+-ATPase as a signal transducer, and $Na_V1.8$ channel.

OPIOID-LIKE RECEPTOR-COUPLED MECHANISM OF $NA_V1.8$ CHANNELS MODULATION

Several unexpected manifestations of morphine action were presented in Chapter **1**. We propose a completely new additional explanation of powerful analgesic effect produced by this substance. It is assumed that the opioid-like receptor-coupled mechanism is also responsible for the analgesic effect of morphine. Of course, the agent runs the well-studied opioidergic system, activation of which leads to pain relief. One terrible disadvantage property intrinsic to opioid receptor agonists, however, does not allow morphine to become an ideal analgesic. This disadvantage is the appearance of multiple negative side effects as a result of its systematic application. It is tempting to speculate that the cause of the adverse side effects of the agent at the molecular level is its ability to activate G proteins coupled to classic opioid receptors. We have found a fundamentally different mechanism of morphine action, the role of signal transducer in which is performed by Na^+,K^+-ATPase of nociceptive neuron [5]. Now it becomes clear that the analgesic effect of morphine is of dual nature: it activates both classic opioid receptors and opioid-like receptors physiologically described in the present book. Our results suggest that a selective agonist of novel opioid-like receptors will be free of negative side effects, since in this case the transducing function would be performed by Na^+,K^+-ATPase and not by G proteins. As it was shown in Chapters **1**-**3**, activation of the Na^+,K^+-ATPase transducing function is a marker of

involvement of opioid-like receptors in modulation of $Na_V1.8$ channels. Identification of the selective agonist of opioid-like receptors which differs essentially from morphine both structurally and physiologically is the important result of our work. This agent is comenic acid, which, unlike morphine, binds selectively only to opioid-like receptors, thus resulting in modulation of $Na_V1.8$ channels responsible for coding of nociceptive signals.

Combined application of the patch-clamp method and quantum-chemical calculations made it possible to clarify the difference between morphine and comenic acid in their receptor-coupled ability to modulate $Na_V1.8$ channels. The latter substance, being of a significantly smaller molecular volume than morphine, specifically activates only opioid-like receptors due to its remarkable property: it can chelate calcium ions from the surrounding physiological medium. Our study on the effects of gamma-pyrone and gamma-pyridone derivatives presented in Chapters **2** and **3** allows to describe the probable characteristics of opioid-like receptor binding pocket and get an insight on molecular structure of the endogenous agonist of these receptors, which is not yet identified.

Four of six studied molecules (substances A, B, E, and F) displayed the ability to modulate $Na_V1.8$ channels, while the other two (substances C and D) were inactive. Our results made it possible to establish that the active substances should bind to the opioid-like receptor being in the form of calcium salt of calcium chelate complex and to consequently formulate the structural criteria determining the possibility for formation of ligand-receptor complexes between gamma-pyridones or gamma-pyrones and the opioid-like receptor: (1) in position 5 of the heterocycle should be present a hydroxyl or methoxy group which is capable, in combination with the carbonyl group in position 4, to chelate Ca^{2+} cation; (2) the second Ca^{2+} cation serves as the counterion at the deprotonated carboxyl or hydroxymethyl group in position 2 of the heterocycle; (3) intercationic distance $r(Ca^{2+}\cdots Ca^{2+})$ may range from 9.4 to 10.0 Å; and (4) Ca^{2+} cations should occupy specific positions with respect to the heterocycle. The major contribution to the energy of ligand-receptor binding of gamma-pyrones and gamma-pyridones is provided by strong ion-ionic interactions between bound calcium cations of the ligand and negatively charged aspartate residues of the opioid-like receptor. It is also found that the nature of the ring heteroatom may influence the ability of ligands to bind to the opioid-like receptor. Substance F, a structural gamma-pyridone analog of inactive gamma-pyrone D, exhibits $Na_V1.8$ channel-modulating effect due to the presence of intramolecular hydrogen bond between the heterocycle nitrogen atom and the oxygen atom of the hydroxymethyl group in position 2 of the pyridone ring, which fixates this substituent in the conformation appropriate for ligand-receptor binding. Several observations are made regarding the structure of opioid-like receptor binding pocket: it is, most

SUBJECT INDEX

A

B

C

D

E

F

G

H

I

K

L

M

N

O

P

Q

R

S

T

V

Z

> This volume deals with the study of molecular ligand-receptor binding mechanisms that modulate $Na_v1.8$ channels responsible for nociceptive signal coding. Investigations of the membrane signaling cascade (opioid-like receptor, Na+, K+-ATPase as a signal transducer, slow sodium channel) reported in this book open new approaches for the development of a novel class of analgesics.
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