Chapter 11

Voltage-Gated Calcium Channel Antagonists: Potential Analgesics for Jejunal Pains

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Additional information is available at the end of the chapter

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Abstract

The significant role of voltage-gated calcium channel (VGCC) L-type antagonists used concomitantly with opioids in attenuation of clinical pain has been confirmed. The aim of this study is to evaluate the comparable effect of intracerebroventricularly (*i.c.v.)* administered diltiazem, nifedipine and/or verapamil – specific antagonists of VGCCs – in the dose of 1.0 and 2.0 mg *in toto* on behavioral signs, clinical symptoms, rumen motor activity and biochemical (plasma cortisol and catecholamine—CA) parameters in sheep that have undergone experimental duodenal distension (DD) and to determine whether voltage-gated calcium channel inhibitors (VGCCIs) exert any anti-nociceptive effects under these conditions. The study was carried out using 24 mature, behind reproductive season crossbred ewes, each weighing 32–42 kg. DD was managed by inclusion and the distension of stretching balloon (having 40 mL of water 39°C temperature—DD40). After 5 min of DD40, the signs were observed: an important augmentation of behavioral nociceptive signs, particularly looking around, defecation, head movement, stretching, grinding, lying down, tachycardia, hyperventilation, inhibition of ruminal contractions (70% approximately, during 15 min) and an increase in plasma catecholamine concentration (over sevenfold increase of epinephrine (E): from 0.24 ± 0.12 in control to 2.98 ± 0.21 mM L^{-1} during 2 h following DD, 2-times norepinephrine (NE): from 1.29 ± 0.23 in control to 2.51 ± 0.30 mM L^{-1} and 124% increase of dopamine (DA): from 0.94 ± 0.02 in control to 2.10 ± 0.35 mM L^{-1}). VGCCI infusion administered 10 min before duodenal distension diminished severity of jejunal nociceptive reactions, for instance, behavioral symptoms, cardiac acceleration, increase in the number of respiration, inhibition of the reticulum and rumen hypomotility, and effortlessly abolished the increasing presence of plasma cortisol and biogenic amines (CA) release. We suggest that the increase and insistence of visceral hyperalgesia stimulate the flow of Ca^{2+} ion flow, provoking neurohormones/neuromediators liberatione and cytoplasmic membrane responsiveness modulation. This result confirmed analgesic effects of VGCCIs L- and/or R-type (nimodipine, lercanidipine, SNX-482) obtained by other authors and also suggests that these channels play a crucial role in the modulation of acute visceral hyperalgesia in sheep and may be a therapeutic target for new drugs.

Keywords: duodenal distension, diltiazem, nifedipine, verapamil, behavioral signs, clinical symptoms, rumen motility, blood plasma cortisol, catecholamine concentration, sheep

1. Introduction

A calcium channel is an ion channel which displays selective permeability to calcium ion (Ca^{2+}). It is sometimes synonymous as voltage-dependent calcium channel (VDCC) [1], although there are also ligand-gated calcium channels (LGCCs) [2]. Voltage-gated calcium channels (VGCCs) are found in excitable cells (e.g., glial cells, muscle, neurons, etc.) [3–6]. There are at least six classes of VGCCs (L-, N-, P/Q-, R- and the T-type channels) that are distributed according to cell type and location, and that may be distinguished by electrophysiological, pharmacological, and structural characteristics. No small organic ligands are clinically available for other than the L-type channels, although there are a number of experimental compounds for the T- and N-type channels [7].

It is known that VGCCs exert a regulatory control of CNS, cardiac, and muscularly activities and that their activity disorders can provide raise to physiopathological cases extending from cardiac and vascular disorders to central nervous system pathologies. Voltage-gated calcium channels inhibitors (VGCCIs) have been applied profitably to treat epilepsy and are arising as probable curative pathways as long as pathology, such as algesia, Parkinson's disease, anxiety, and addiction [8]. Therefore, calcium channels can be drug targets for nervous system diseases, and potential challenges and opportunities for the development of new clinically effective calcium channel inhibitors [8].

L-type VGCCs are located in neuronal cells, dendrites, spinal cord, adrenal gland, skeletal cardiac and smooth muscles, and many other locations [9–14]. L-type calcium currents typically require strong depolarization for their activation and are blocked by different antagonists (VGCCIs) including dihydropyridines (nifedipine), benzothiazepines (diltiazem), and phenylalkylamines (e.g., verapamil). VGCCIs are a class of drugs that disrupts the movement of calcium ions through calcium channels. These substances, by relaxing the smooth muscle tone, are commonly used to treat high blood pressure (hypertension), migraines, *angina pectoris*, Raynaud's disease, and also cluster headaches [9, 14]. In palliative medicine, they are used as analgesic drugs and in veterinary, they are used to treat experimental duodenal acute pain (colic) in sheep [15, 16]. Since high density of these channels are found in sinoatrial and atrioventricular nodes, VGCCIs decrease impulse conduction through these nodes and are used as antiarrhythmic agents.

The mode of action of verapamil similarly to diltiazem and nifedipine, is based on binding with the largest subunit α_1 of Ca^{2+} channels. This subunit incorporates the conduction pore, voltage sensor, gating apparatus, and several regulation sites, e.g., by second messengers, drugs, and toxins. VGCCIs inhibit Ca^{2+} ions influx to the cells, which are the main Ca^{2+} currents

in muscle and endocrine cells initiating many activities, such as gene expression, muscle contraction (excitation-contraction coupling), hormone secretion, neurotransmitter release, cell growth and regulation, neurons migration, cell damage, and death or finally cell survival [17].

Acute intestinal distension ("colic"), similarly as functional gastrointestinal disorders, inflammatory bowel disease or irritable bowel syndrome causes visceral hypersensitivity and may produce persistent pain [17, 18]. Visceral pain is described as pressure-like, intermittently squeezing or cramp, not well localized, vague in character, and difficult for patients to describe [19]. Visceral pain is frequently accompanied by nausea, sweating, defecation, vocalization, grinding, head movement, hyperventilation, hypertension, tachycardia, hypercortisolemia, and hypercatecholaminemia (**Table 1**).

Accompanying symptoms	**0–5**			**5–10**		**10–15**			**25–30**		**55–60**		**120 min**	
	DD	**1**	**2**	**3**	**DD**	**1**	**2**	**3**	**DD**	**1**	**2**	**3**	**DD**	**1**	**2**	**3**	**DD**	**1**	**2**	**3**
Inhibition ruminal activity	4+	−	+	−	4+	−	±	−	3+	2	+	+	−	−	−	−	−	−	±	−	−	−	−
Looking around	3+	±	±	±	2+	±	±	±	+	+	+	+	−	−	−	+	−	−	−	−	−	−	−
Defecation	3+	−	−	−	+	−	±	−	−	±	−	±	−	−	±	+	−	−	−	−	−	−	−
Head movements	3+	−	−	−	2+	±	±	±	−	−	−	−	−	−	±	+	−	−	−	−	−	−	−
Stretching	2+	−	−	−	−	+	−	−	−	−	+	±	−	−	−	−	−	−	−	−	−	−	−
Grinding	2+	±	±	±	±	−	+	+	−	±	±	±	−	−	−	−	−	−	−	−	−	−	−
Lying down	2+	−	−	−	−	+	−	−	−	±	±	−	−	−	−	−	−	−	−	−	−	−	−
Bleating	+	−	−	−	−	+	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−
Tachycardia	4+	±	3+	3+	±	4+	−	±	4+	−	3+	±	3+	−	±	±	3+	−	±	±	3+	−	−	−
Hyperventilation	4+	±	−	±	3+	−	−	±	4+	−	−	±	3+	−	−	−	3+	−	−	−	3+	−	−	−

1, Diltiazem + DD40; 2, Nifedipine + DD40; 3, Verapamil + DD40.

Table 1. The effect of duodenal distension (DD40) on the ruminal motility (inhibition in % 5 min^{-1} in comparison to the control values) [10] and behavioral symptoms (number·5 min^{-1}) in sheep before and after voltage-gated calcium channels inhibitor pretreatment at a dose of 1 or 2 mg *in toto* (i.e. 25 or 50 $\mu g \cdot kg^{-1}$ B.W.; n = 6).

Gastrointestinal sensory system consist intrinsic (enteric) sensory afferents and extrinsic (vagus, spinal cord, pelvic) afferents. Intrinsic sensory system functions independently of the CNS. Enterochromaffin cells within mucosa and enteroendocrine cells release 5-HT, CCK, orexin, and leptin which modulates and regulates motor activity of intestine [20, 21]. The submucosal enteric plexus and myenteric plexus have a high degree of synaptic interactions (enteric nervous system or a "gut brain"), which can be either inhibitory or stimulatory for the purpose of regulating gastrointestinal motility and peristalsis [22, 23].

Mechanisms of the reception, transduction, transformation and modulation of nociceptive stimulus, and reaction diminishing response on nociception are regulated by afferent systems to CNS and efferent systems from CNS "stimulating" reaction, but quenching (pain-gated).

Several data show antinociceptive/antistressoric effects of organic Ca^{2+} inhibitors of L-VGCCs in acute duodenal pain of sheep [16, 20]. These inhibitors potentiate the analgesic action of κ-opioidergic receptor agonists [15], as well as morphine by decreasing opioids' tolerance [24]. It was also shown by Bongianni et al. [25] that VGCCIs suppress not only metabolic but also behavioral expression of the morphine withdrawal syndrome. In experiments performed on mice, it was shown that verapamil blocked amphetamine and also physostigmine induced footshock-induced aggression [26]. It was postulated by Michaluk et al. [27] that VGCCIs show antinociceptive properties; but they also change the territorial behavior of animals [28] and conspecific aggression in fish [29]. Such effects were probably caused by the inhibition of Ca^{2+} entry into neurons, preventing the appearance of synaptic vesicles in axon terminal, and release of neurotransmitter into the synaptic cleft. Davis and Bauer [14] have shown in experiments performed on rats, that activation of L-VGCCs are necessary for the long-term retention of fear excitation.

A duodenal and/or colonic distension method, provoking jejunal pain, stimulates hypothalamic-pituitary-adrenal-cortical (HPA) and sympatico-adrenal system (SAS), pathways that revealed as an increase in cortisol and CA in blood plasma [18, 20]. A different role of L-type antagonists for VGCC has been previously identified in different types of experimental and clinical pain in man and animals. Present study examined comparative role of VGCC blockers from different chemical groups—diltiazem, nifedipine, and verapamil administered *i.c.v.* in the same four different doses (0.25, 0.5, 1.0 and/or 2.0 mg *in toto*)—to estimate the comparable effect on the development of pain-related symptoms, clinical signs, plasma cortisol and catecholamine level, and the inhibition of ruminal motor activity caused by 5 min lasting mechanical duodenal distension (DD) in the sheep.

2. Material and methods

2.1. Preparation of animals

Experiment was carried out on 24 mature crossbred ewes, Polish merino sheep weighing 32–42 kg B.W., being *in anoestrus* period accordingly to the earlier described methods [16, 18, 20]. Food was removed 24 hours prior to the experiment. Analgesia was initiated by *i.m.* ketamine (Calypsovet, 20 mg kg^{-1} B.W., Gedeon Richter, Budapest, Hungary) administration, and 15 min later, *i.v.* infusion of pentobarbital anaesthesia in the dose of 20 mg kg^{-1} B.W. (Vetbutal, BIOWET, Pulawy, Poland) was performed. During unconsciousness, a T-shaped silicon cannula (inside diameter of 21 mm) was inserted into the duodenum (12–15 cm from pylorus). Secondly, an identical cannula was inserted into the dorsal sac of the rumen, using techniques described previously [30] on all animals. Simultaneously, under the same general anaesthesia/analgesia, a permanent stainless steel cannula, 29 mm length and 2 mm in diameter (guide cannula), was inserted into the lateral cerebral ventricle (on the left and/or the right side) of the brain, 10 mm above the bregma and 5 mm laterally from the midline suture using stereotaxic method described by Sorraing et al [31]. After recovery from surgery, the animals were

placed in metabolic cages at constant temperature (18–20°C) for at least 14 days prior to beginning of the experiment [20].

Investigations were performed in four steps (groups, each of six animals for every drug). Every exercise was carried together on two unfed animals, placed in separate boxes at seven days interruption. Blood collection was performed 30 min prior to investigation, in 0 time and 5, 10, 15, 30, 60, and 120 min (**Figure 1**).

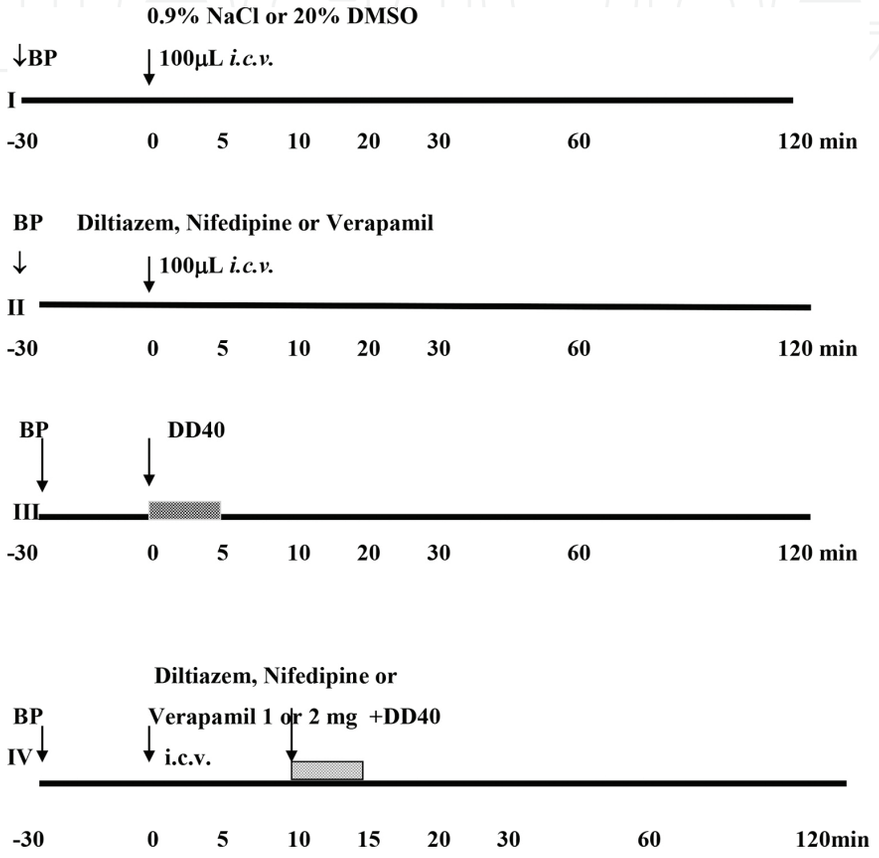

Figure 1. Experimental timelines for the four test group and blood sampling: Control: 0.9% solution NaCl or 20% DMSO—100 μL *i.c.v.* Diltiazem, nifedipine, or verapamil—1.0 or 2.0 mg *in toto* in 100 μL of 0.9% NaCl or 20% DMSO *i.c.v.* DD40—duodenal distension—40 mL water (temp. = 39°C) placed into rubber balloon. Duodenal distension + drug treatment. The time at which the intraduodenal balloon was inserted is marked with the letters: BP.

In the first experimental group, a 60 min recording of the ruminal motility was performed in each animals (n = 6) receiving 100 μL of 20% DMSO (control for nifedipine) or 0.9% NaCl (control for diltiazem and/or verapamil), during 1 min infusion (20 min after the 1st venous blood collection) into the lateral ventricle of the brain (*i.c.v.*); the rumen contraction was registered for 90 min (schema of the blood collection, it was respectively to **Figure 1**).

The second group of sheep (n = 6) were subjected to VGCCIs treatment alone, after 60 min control recording of rumen motility. Every animal received each dose of the substance (with 7 days interval). After the second collection of the venous blood, the sheep were *i.c.v.* given a 1 min lasting infusion of 100 μL of nifedipine in 20% DMSO solution (diltiazem or verapamil

in 0.9% NaCl solution) in a dose of 0.25 mg in the first, 0.5 in the second, 1.0 mg in the third or 2.0 mg *in toto* (6.25, 12.5, 25.0 or 50.0 μg kg^{-1} B.W.) in 4 weeks and then the registration was maintained for the next 90 min.

In the third group of sheep (n = 6), after 30 min of control registration of the rumen motility, a rubber balloon (10 cm long) was inserted into the duodenum *via* the duodenal fistula. After placing the balloon in the jejunum, soon after the 2nd blood collection (0 time), the balloon was filled with 40 mL of warm water (DD40) and the distension was maintained for 5 min [28]. Then, the recording of ruminal contractions was continued for 60–90 min. Ten minutes before DD40, each animal received *i.c.v.* infusion of 100 μL of solvents for the drugs tested (**Figure 1**).

In the fourth group of sheep (n = 6), after 30 min of control registration of the rumen motility, a rubber balloon (10 cm long) was inserted into the duodenum and 30 min after the animals received the 100 μL *i.c.v.* infusion of diltiazem, nifedipine (in 20% DMSO solution) or verapamil at a dose of 0.25 mg in the first, 0.5 mg in the second, 1.0 in the third or 2.0 mg *in toto* in the 4th week (the same mode it was used for diltiazem, nifedipine and verapamil experimentation). After 10 min of the diltiazem, nifedipine or verapamil, 1-minute infusion duodenum was distended for 5 min with the balloon containing 40 mL of water (DD40) at body temperature. After the 5 min distension was over, the recording was continued for 60–90 min.

Experimental procedure lasted for 10 months. The doses of 1.0 and 2.0 mg diltiazem, nifedipine or verapamil *in toto* were effective in premedication contra DD40 only.

2.2. Mechanography

The ruminal contractions were analyzed using the electronic tensometric recorder PIT 212 (COMT, Bialystok, Poland). The analysis of mechanograms and calculations of results were performed similarly as in a case of electromyographic recording [32]. The number of the rumen motor activity was determined by the frequency on mechanograms, with 5 min intervals before and after the DD40.

2.3. The estimation of blood cortisol and catecholamine (CA) levels

Blood samples for the analysis of CA estimation were collected from the jugular external vein (according to a scheme described above—**Figure 1**). Blood samples were placed in 10 mL test tubes containing reduced glutathione (0.05 mM). The plastic tubes were maintained on ice, and after the centrifugation, plasma was stored at −80°C, until the beginning of the analytical process. The detection of CA levels was performed by radioimmunoassay using REA kits (CATECHOLA, Czech Republic). The sensitivity of this method was for E, 0.37, for NE, 0.53, and for DA, 0.85 nM L^{-1}. The intra serial error for E was of 3%, for NE 4.2%, and for DA 6.1%, whereas the error among the series was of 4.2, 7.4, and 6.6%, respectively.

Cortisol levels were detected by radioimmunoassay (RIA), according to previous experiments [18]. The mean intra and inter assay of the method was of 9.5 pg, for a sample of 10 μL (ORION DIAGNOSTICA, Espoo). Δ_{max} concentration for each hormone was the difference between the basal concentration and the highest concentration measured.

2.4. The determination of cardiac and ventilation rates

The heart and respiratory rates were measured by determining the number of heart frequencies, as well as by observing the respiratory thoracic movements, using the stethoscope for 1 min. These estimations were detected out by the same person prior to blood test for analysis, according to earlier article [20].

The lack of effect of solvents was determined in preliminary experiments [29, 32].

2.5. Statistical analysis

Statistical significance of the results was carried out through the comparison of control values with those obtained after mechanical distension (duodenal distension—DD40), as well as after VGCCIs premedication and concurrent DD40, using a multifactorial analysis of variance (ANOVA). The statistical significance of the results was detected with a post hoc Tukey-Kramer test; the results are shown as a mean ± SEM. A p value, less than 0.05 was considered statistically significant in all tests.

The researches were performed according to the rules of the Principles of Laboratory Animal Care (NIH publication no. 86-23, revised 1985), as well as the specific national laws on protection of animal (National Law for Animals Protection – 1997, Dz. U.23 XI; Permission of 3rd Local Ethical Commission No. 9/2001 issued 11.01.2001).

3. Results

The influence of DD on behavioral signs, clinical symptoms, reticulo-ruminal contraction and blood plasma cortisol, and CA level was investigated. Before the physiological experimentation, no alteration was observed neither in the physiological behavior of two animals, simultaneously tested in individual cages, nor in the motoric and behavioral response to environmental factors. The mean cardiac frequency was 75.2 (±5.11) and the number of breathing was 35.6·min^{-1} (±4.21), and the reticulo-ruminal frequency was 6.45 ± 0.75 c × 5 min^{-1} in 30 min, before DD. After the implantation of an empty rubber balloon into the duodenum, the observed changes were NS in animal behavior or in the heart beat (70.5 beats·min^{-1} (±8.2)), respiration frequency (42.1 ± 6.35·min^{-1}, **Table 1**) and reticulo-ruminal contractions (6.12 ± 0.28 c·5 min^{-1}). Five-minute duodenal distension induced highly statistically significant alterations in the behavioral signs of animals [10].

3.1. The effect of DD on the behavioral and clinical symptoms, blood plasma cortisol, and/or catecholamine level

Duodenal distension by 40 mL of warm water resulted in a significant increase in the behavioral pain responses: motility, bleating, teeth grinding, prostration, wetting, defecation, tachycardia (from 60 ± 3 beats·min^{-1} to 86 ± 6.2 beats·min^{-1}), hyperventilation (from 36.3 ± 3.6 number·min^{-1} to 50.3 ± 4.5 number·min^{-1}), inhibition of reticulo-rumen contractions rate (from 6.15 ± 0.54 c × 5 min^{-1} in control to 1.09 ± 0.33 c × 5 min^{-1} during DD and to 1.35 ± 0.52

c × 5 min^{-1} 10 min after DD (**Figure 2**; p = 0.001), from −82.2% to −78.0% during 15–20 min; a significant increase in plasma cortisol concentration from 10.51 ± 2.66 ng·mL^{-1} in control to 24.72 ± 8.25 ng·mL−1 during DD and to 34.44 ± 5.46 ng·mL^{-1} (p ≤ 0.01) 10 min after DD (**Figure 3**); a statistically significant increase of plasma catecholamine concentration (over seven-fold increase of E from 0.34 ± 0.12 nM·L^{-1} in control to 2.87 ± 0.65 nM L^{-1}, during 2 hours following the DD (**Figure 4**); 100% NE—from 1.29 ± 0.23 nM·L^{-1} in control to 2.32 ± 0.24 nM·L^{-1}—120 min after DD (**Figure 4**) and 126% increase of DA from 0.93 ± 0.02 nM L^{-1} in the control to 2.33 ± 1.16 nM L^{-1}, 120 min after DD) (**Figure 4**).

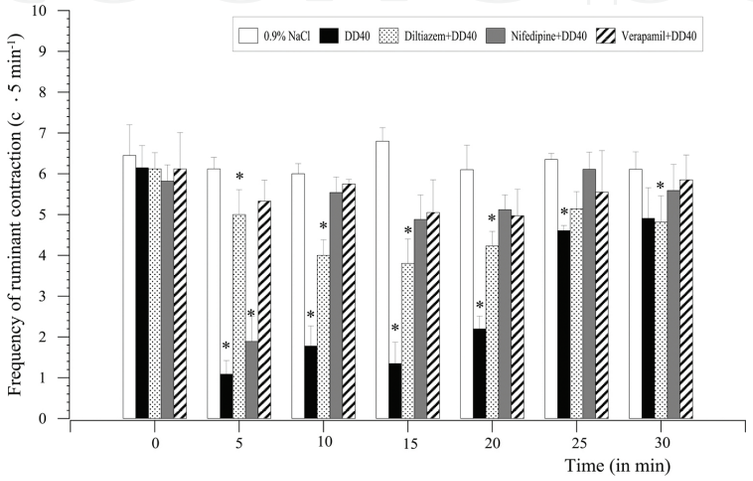

Figure 2. Influence of *i.c.v.* 1 min infusion after 10 min pretreatment of different voltage-gated calcium channels inhibitor (diltiazem, nifedipine or verapamil) in different doses (1.0 or 2.0 mg/animal) per number of reticulo-ruminal contractions (c × 5 min^{-1}) in sheep in comparison with the group with duodenal distension, DD40 (x ± SEM, n = 6). Mean values of results obtained from the same blood collection (time point) with different superscript sign p ≤ 0.001–0.05 level in comparison to DD40 value.

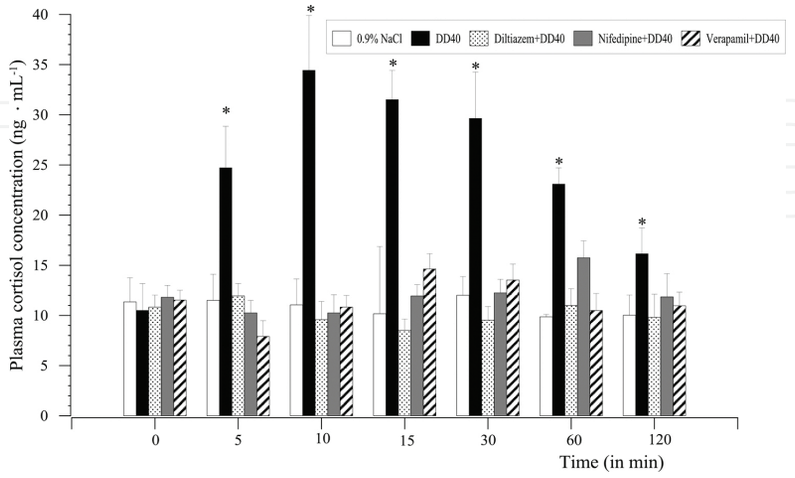

Figure 3. Comparative analysis of 10 min premedication influence with *i.c.v.* diltiazem, nifedipine, and/or verapamil (in doses 1.0 and/or 2.0 mg/animal) and DD40 on plasma cortisol concentration in sheep, in comparison to DD40 value at p ≤ 0.001–0.05 (x ± SEM, n = 6).

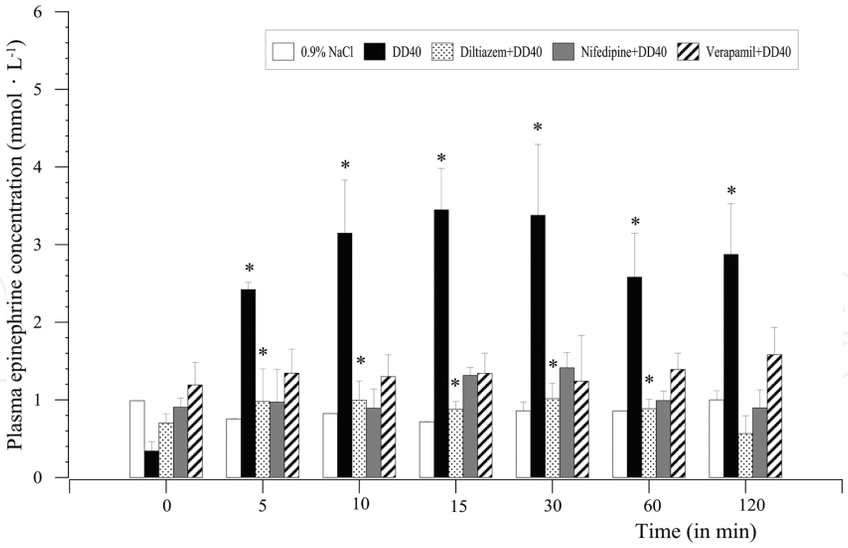

Figure 4. Comparative analysis of duodenal distension and premedication with different diltiazem, nifedipine and verapamil doses (1.0 or 2.0 mg/animal) on plasma epinephrine concentration in comparison with DD40 (x ± SEM, n = 6, p ≤ 0.001–0.05). Mean values of results obtained from the same blood collection (time point).

3.2. The influence of VGCCIs premedication on the behavioral changes, clinical symptoms, rumen motility, plasma cortisol, and catecholamine level in animals with/without DD

I.C.V. infusion of 1 min diltiazem, nifedipine or verapamil in doses of 0.25, 0.50, 1.0 or 2.0 mg *in toto*, did not have any significant influence on the behavioral and clinical symptoms (**Table 1**), rumen contractions count (**Figure 2**), cortisol (**Figure 3**), and CA's level in blood plasma (**Figures 4–6**).

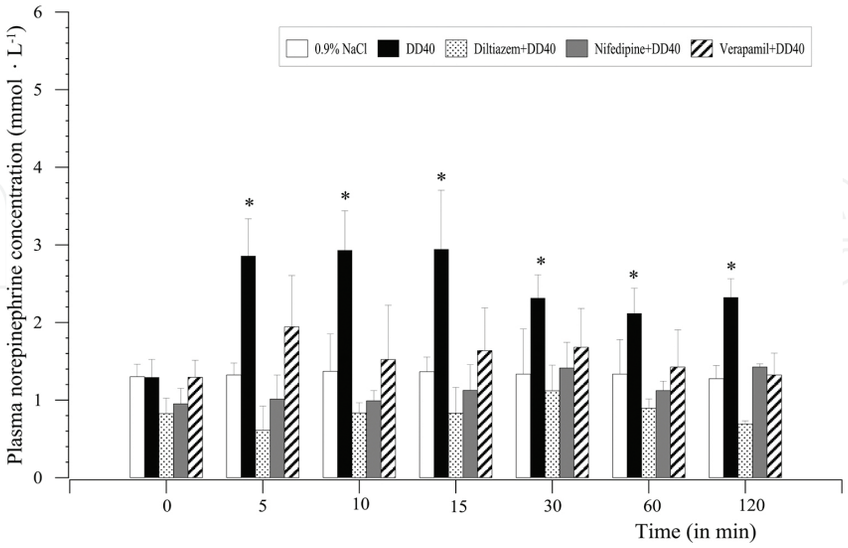

Figure 5. Comparative analysis of duodenal distension and premedication with different diltiazem, nifedipine and verapamil doses (1.0 or 2.0 mg/animal) on plasma norepinephrine concentration in comparison with DD40 (x ± SEM, n = 6, p ≤ 0.001–0.05). Mean values of results obtained from the same blood collection (time point).

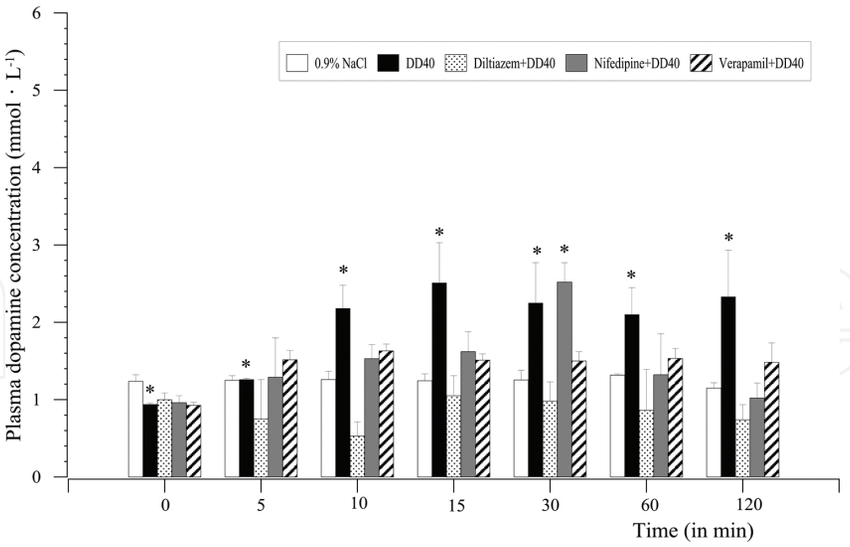

Figure 6. Comparative analysis of duodenal distension and premedication with different diltiazem, nifedipine and verapamil doses (1.0 or 2.0 mg/animal) on plasma dopamine concentration in comparison with DD40 (x ± SEM, n = 6, p ≤ 0.001–0.05). Mean values of results obtained from the same blood collection (time point).

I.C.V. infusion of diltiazem, nifedipine, and verapamil (1.0 or 2.0 mg *in toto*), 10 min before the DD inhibited and/or completely attenuated the beginning of clinical symptoms of jejunal nociception, provoked by duodenal distension (**Table 1**). In control animals before premedication, intense acceleration of cardiac beats was observed (mean from 70 to 102 beats·min^{-1}) and in the animals treated with diltiazem or verapamil, it was decreased to 63–86 beats·min^{-1}; after nifedipine premedication, the DD caused an increased cardiac frequency from 68 to 90 beats·min^{-1}. Respiration frequency was 50 and 34·min^{-1}, respectively.

Drugs	Control	5 min	10 min	15 min	20 min	25 min	30 min
0.9% NaCl	6.45 ± 0.75	6.12 ± 0.28	6.00 ± 0.25	6.80 ± 0.33	6.10 ± 0.60	6.35 ± 0.15	6.11 ± 0.43
DD40	6.15 ± 0.54	1.09 ± 0.33*	1.78 ± 0.49*	1.35 ± 0.52*	2.20 ± 0.31*	0.61 ± 0.12*	4.91 ± 0.75
Diltiazem + DD40	5.00 ± 0.61*	4.00 ± 0.38*	3.80 ± 0.60*	4.23 ± 0.36*	5.14 ± 0.42	4.82 ± 0.64	6.12 ± 0.40
Nifedipine + DD40	5.82 ± 0.45	1.89 ± 0.81*	5.54 ± 0.23	4.88 ± 0.62	5.12 ± 0.74	6.11 ± 1.11	5.59 ± 1.22
Verapamil + DD40	6.12 ± 0.89	5.33 ± 0.51	5.75 ± 0.11	5.05 ± 0.80	4.97 ± 0.65	5.55 ± 1.02	5.85 ± 0.61

Value are mean ± SEM of 6 sheep, and indicate significant difference corresponding from control group.
ẍ ± SEM, n = 6.
*p ≤ 0.001–0.05.

Table 2. Frequency of ruminal contraction of the five groups (control, DD40, diltiazem + DD40, nifedipine + DD40, verapamil + DD40) during the course of the experiments.

VGCCIs premedication caused that inhibition of rumen frequency after 5 min DD, decreasing from average 6.12 ± 0.40 to 5.00 ± 0.61, in 5 min, and 4.00 ± 0.38 c × 5min^{-1}, in 10 min, after DD,

but not from 6.15 ± 0.54 in control to 1.09 ± 0.33 c × 5 min^{-1} after DD (**Table 2**, **Figure 2**). Plasma cortisol concentration changed from 10.83 ± 1.19 in control to 11.95 ± 1.25, during (NS), and 9.53 ± 1.36 (NS) 30 min, after DD, but not increased from 10.51 ± 2.66 in control to 24.72 ± 8.25, during DD, and 34.44 ± 5.46 (+227.7%) 10 min, after DD ($p<0.001$, **Figure 3**). Diltiazem premedication caused that increase of plasma CA concentration after 5 min DD, decreased E from average +606.41% in control to +23.02%, during 120 min after DD (**Figure 4**); NE from +120.95% in control to −21%, during 120 min after DD (**Figure 5**) and DA from +124.2% in control to −24,8%, during 120 min after diltiazem premedication (**Figure 6**).

Nifedipine *i.c.v.* premedication caused that inhibition of rumen frequency after 5 min DD, decreased from average 5.82 ± 0.45 to 1.89 ± 0.81 (−69%) in 5 min only, during DD, and 5.54 ± 0.23 (−5.2%) 10 min, after DD, but not from 6.15 in control to 1.78 c × 5 min^{-1} (−71.%), after DD (**Figure 2**). Nifedipine premedication diminished the increase in plasma cortisol concentration from 11.81 ± 1.13 ng L^{-1} (NS) in control to 10.25 ± 1.65 ng · L^{-1} (NS) in 10.00 and to 11.85 ng L^{-1} 120 min after DD (**Figure 3**). Premedication by 1 min *i.c.v.* nifedipine infusion caused that increase of plasma catecholamine concentration, after 5 min DD, statistically significantly decreased from average 0.34 to 2.98 nM L^{-1} (+767.8%) in DD and from 0.91 in control to 1.08 nM L^{-1} (+23%) to E, from +98.5 to +23.7%, to NE, and from +124.2 in control to 61.3% to DA, during 120 min after DD (**Figures 4–6**).

Verapamil *i.c.v.* premedication by 1 min infusion caused that inhibition rumen frequency after 5 min DD, decreased from 6.12 ± 0.89 to 5.38 ± 0.53 (−12.1%) during 30 min after DD and by average 44.7% in comparison to DD only (**Table 2**). In the same time, verapamil premedication caused that increase of plasma cortisol concentration after 5 min DD, decreased from average 11.53 ± 0.98 in control to 7.91 ± 1.58 (−36.1%; $p<0.05$) during DD and 11.39 ± 1.48 ng·L^{-1} (NS) during 120 min after DD and by −153% ($p<0.001$) in comparison to DD group (**Table 3**).

	Control	5 min	10 min	20 min	30 min	60 min	120 min
0.9% NaCl	11.35 ± 2.40	11.50 ± 2.61	11.05 ± 2.58	10.17 ± 6.71	12.01 ± 1.86	9.87 ± 0.25	10.02 ± 2.02
DD40	10.51 ± 2.66	24.72 ± 8.25*	34.44 ± 5.46*	31.52 ± 2.91*	29.65 ± 4.61*	23.10 ± 1.61*	16.16 ± 2.56*
Diltiazem + DD40	10.83 ± 1.19	11.95 ± 1.25	9.58 ± 1.81	8.52 ± 1.13	9.53 ± 1.36	10.99 ± 1.68	9.8 ± 2.31
Nifedipine + DD40	11.81 ± 1.13	10.25 ± 1.51	10.25 ± 1.17	11.94 ± 1.65	12.25 ± 1.39	15.75 ± 1.69	11.85 ± 1.35
Verapamil + DD40	11.53 ± 0.98	7.91 ± 1.58*	10.83 ± 1.17	14.63 ± 1.53	13.53 ± 1.58	10.49 ± 1.69	10.96 ± 1.36

*$p \leq 0.001$–0.05.

Table 3. Comparative analysis *i.c.v.* diltiazem, nifedipine, or verapamil (in the doses 2 mg *in toto*) premedication influence and DD40 on plasma cortisol concentration changes in sheep.

Verapamil *i.c.v.* premedication caused that plasma epinephrine after 5 min DD increased, but nonsignificantly from average 1.19 ± 0.29 in control to 1.36 ± 0.59 (+14.7%), during 120 min after DD, but not from 0.34 ± 0.12 in control to 2.99 ± 0.81 nM L^{-1}, e.g., +767.8% ($p<0.001$), during

120 min after DD (**Table 4**). Decrease of epinephrine plasma concentration by verapamil premedication was 753.08%.

Verapamil *i.c.v.* premedication caused that increase of plasma norepinephrine concentration after 5 min DD, increased from average 1.29 ± 0.22 in control to 1.58 ± 0.84 nM L^{-1}, during 120 min after DD, but not from 1.29 ± 0.23 in control to 2.50 ± 0.42 nM L^{-1} (+98.56%), during 120 min after DD. Decrease in norepinephrine concentration by verapamil premedication was 75.71%.

Catecholamine	**Time (in min)**						
	0	**5**	**10**	**15**	**30**	**60**	**120**
Epinephrine							
0.9% NaCl (100 μL)	0.98 ± 0.00	0.75 ± 0.00	0.82 ± 0.00	0.71 ± 0.00	0.85 ± 0.11	0.85 ± 0.00	0.99 ± 0.12
DD40	0.34 ± 0.12	2.42 ± 0.09	3.15 ± 0.68	3.45 ± 0.53	3.38 ± 1.82	2.58 ± 1.13	2.87 ± 0.65
Diltiazem + DD40	0.70 ± 0.12	0.98 ± 0.42	0.99 ± 0.25	0.88 ± 0.10	1.01 ± 0.20	0.88 ± 0.12	0.56 ± 0.23
Nifedipine + DD40	0.90 ± 0.12	0.97 ± 0.14	0.89 ± 0.25	1.31 ± 0.58	1.41 ± 0.38	0.99 ± 0.18	0.90 ± 0.41
Verapamil + DD40	1.19 ± 0.29	1.34 ± 0.31	1.30 ± 0.28	1.34 ± 0.26	1.24 ± 0.59	1.39 ± 0.21	1.58 ± 0.35
Norepinephrine							
0.9% NaCl (100 μL)	1.30 ± 0.16	1.32 ± 0.15	1.37 ± 0.48	1.36 ± 0.19	1.33 ± 0.58	1.33 ± 0.44	1.28 ± 0.17
DD40	1.29 ± 0.23	2.85 ± 0.48	2.93 ± 0.51	2.94 ± 0.76	2.31 ± 0.30	2.11 ± 0.32	2.32 ± 0.24
Diltiazem + DD40	0.82 ± 0.20	0.61 ± 0.31	0.83 ± 0.13	0.83 ± 0.33	1.12 ± 0.33	0.89 ± 0.12	0.69 ± 0.04
Nifedipine + DD40	0.95 ± 0.06	1.01 ± 0.14	0.99 ± 0.28	1.12 ± 0.38	1.41 ± 1.22	1.12 ± 0.72	1.43 ± 1.23
Verapamil + DD40	1.29 ± 0.22	1.94 ± 0.66	1.52 ± 0.42	1.63 ± 1.12	1.68 ± 1.10	1.42 ± 0.48	1.32 ± 0.24
Dopamine							
0.9% NaCl (100 μL)	1.23 ± 0.08	1.24 ± 0.06	1.26 ± 0.10	1.24 ± 0.09	1.25 ± 0.12	1.31 ± 0.01	1.15 ± 0.07
DD40	0.93 ± 0.02	1.26 ± 0.01	2.18 ± 0.30	2.51 ± 0.52	2.25 ± 0.52	2.10 ± 0.35	2.33 ± 1.16
Diltiazem + DD40	0.99 ± 0.09	0.75 ± 0.51	0.53 ± 0.18	1.05 ± 0.26	0.98 ± 0.25	0.86 ± 0.53	0.74 ± 0.19
Nifedipine + DD40	0.96 ± 0.02	1.29 ± 0.12	1.53 ± 0.42	1.62 ± 0.28	2.52 ± 1.41	1.32 ± 0.19	1.02 ± 0.32
Verapamil + DD40	0.92 ± 0.04	1.51 ± 0.12	1.63 ± 0.09	1.51 ± 0.08	1.50 ± 0.12	1.53 ± 0.13	1.48 ± 0.25

Table 4. Comparative analysis *i.c.v.* diltiazem, nifedipine, and verapamil (in the dose of 1.0 or 2.0 mg *in toto*) premedication influence and DD40 on concentration epinephrine, norepinephrine and dopamine plasma level changes in sheep in comparison to the control values (ẍ ± SEM; n = 6).

I.C.V. verapamil premedication caused that plasma dopamine concentration after 5 min DD increased from 0.92 ± 0.04 in control to 1.52 ± 0.13 nM L^{-1}, during 120 min after DD (+65.04%),

but not from 0.93 ± 0.02 in control to 2.33 ± 1.16, during 120 min after DD (+126.3%; $p<0.001$, **Table 4**). Decrease in dopamine concentration by verapamil premedication was 61.26% ($p<0.01$).

4. Discussion

The results of this experiment showed that 1 min diltiazem, nifedipine, and/or verapamil (VGCCIs) *i.c.v.* infusion in doses 0.25, 0.5, 1.0, or 2.0 mg *in toto*, given 10 min before DD, decreased the intensity of visceral nocifensive responses, such as behavioral changes, tachycardia, hyperventilation, reticulo-ruminal motility inhibition, and efficiently prevented the appearance of cortisol and catecholamine concentration in the blood plasma, after two higher doses. It was established that these results revealed that the development and persistence of acute duodenal pain depends on the activation of Ca^{2+} ion flux, leading to neurohormones release and modulation of membrane excitability. It seems that VGCCIs given *i.c.v.* 10 min prior to DD, which was evoked by the darting pain, blocked specific receptors $\alpha(1)$ subunits of voltage-gated calcium channels in effector tissues, attenuate depolarization of cellular membranes, and liberation of neurotransducers important for pain perception in small ruminants. The confirmed analgesic effect of L-type VGCCIs proposes that these L-type VGCCs play a crucial role in the modulation of acute experimental visceral pain in sheep. The important significance of VGCC L-type inhibitors, applied together with opioids in weakness of clinical nociception, have been revealed by Gullapalli and Ramarao [33], that L-type channel modulation by 1,4-dihydropyridines (nimodipine and lercanidipine) potentiates kappa-opioid receptor agonist induced acute analgesia and inhibits the development of tolerance in rats using the tail-flick test. Nimodipine (1 $mg \cdot kg^{-1}$; *i.p.*) and lercanidipine (0.3 $mg \cdot kg^{-1}$; *i.p.*) used in this study produced no tail-flick analgesia, but administered that in these doses, 15 min prior analgesic doses of selective kappa-opioid agonists (U 50,488, PD 117,302 and U 69,593) significantly potentiated the analgesia produced by three kappa-opioid receptor agonists. These results strongly suggested a functional role of L-type Ca^{2+} channels in the regulation of pain sensitivity and mechanism of kappa-opioid analgesia. Last results by Qian et al. [17] suggest also, an important role of VGCCs in rat visceral hypersensitivity by 2,4,6-trinitrobenzenesulfonic acid provoked car nimodipine and SNX-482 prevented it.

In our study, saline and 20% DMSO *i.c.v.* infused during 1 min in volume of 100 μL did not change the ruminal motor activity during 30 min before DD40 and after introduced empty balloon, it was 1.38 ± 0.14 $c \cdot min^{-1}$. The results obtained were nonsignificant in comparison with the results obtained after the intraduodenal balloon placed and no influencing on the interpretation of the results obtained. Mechanical duodenal distension by balloon 10 cm in length with 40 mL of warm water (DD40) provoked during and after 5 min total inhibition of spiking activity [30, 32] or contraction of the rumen and duodenum during 8–12 min ($p \leq 0.01$, **Figure 2**), approximately 85%. These effects, lasting 20 min after DD termination (average 47.3%) were statistically significant ($p \leq 0.05$) in comparison to the control values (**Figure 2**). During 5 min episode of DD40, only one to two ruminal contractions were registered in six

animals tested. Singular contraction recurred after terminations of DD40 immediately, but their number did not exceed the values of control contractions.

It is known that DD40 provoked stimulation of behavioral signs, clinical symptoms, and statistically significant increase in plasma cortisol and catecholamine concentrations (**Table 1**, **Figures 3–6**). *I.C.V.* premedication, by VGCCIs attenuated ruminal motor inhibition by 5 min episode DD, provoked during 30 min average 39.4%, e.g., from 56.8% after DD to 24.2, 18.1 and 9.6% after diltiazem, nifedipine, and verapamil premedication, respectively. The most preventing for the 5 min DD inhibitory influence on ruminal motility was verapamil in comparison to the control values. All the VGCCIs in premedication use inhibited statistically significant behavioral signs, such as looking around, defecation and/or urination, head movements, lying down, and clinical symptoms, such as tachycardia and hyperventilation by 120 min after DD persistent (**Table 1**).

Five-minute DD episode increased the plasma cortisol concentration average for 153% during 120 min after DD, in comparison to the control values. Ten-minute VGCCIs premedication diminished the plasma cortisol release by an average of 139.5 % (diltiazem), and 141% (nifedipine and verapamil) ($p \leq 0.001$; **Table 3**, **Figure 3**) respectively.

Five-minute DD episode increased the plasma catecholamine concentration average: E 768%, NE 98.5%, and DA 124% during 120 min after DD. Diltiazem, nifedipine, and verapamil minimized the increase of plasma catecholamine concentration, which was caused by visceral pain, provoked by duodenal distension. Average of catecholamine release inhibition by VGCCIs were for E—773.3%, NA—90%, and DA—90.2%, during 120 after 5 min DD episode ($p \leq 0.001$; **Table 4**, **Figures 4–6**). The most anticatecholaminergic activity was detected for diltiazem. In our study, we found that all the VGCCIs—whatever their chemical origin—in premedication attenuated vegetative signs and clinical symptoms, HPA and SAS stimulation axes caused by acute 5 min duration nocifensive factor (duodenal distension) in sheep.

This confirms the results obtained by Qian et al. [17] that $Ca_v1.2$ and $Ca_v2.3$ channels in colonic primary sensory neurons play an important role in visceral inflammatory hyperalgesia, which may be a potential therapeutic target, because L-type and R-type selective colonic channel blockers may block calcium currents which are importantly increased in colonic dorsal root ganglion (DRG) neurons of 2,4,6-trinitrobenzenesulfonic acid-treated rats in comparison with control animals. The author cited above concluded that L-type channel antagonist (nimodipine) and R-type channel antagonist (SNX-482) attenuates visceral pain in 2,4,6-trinitrobenzenesulfonic acid intrathecal injected. The results obtained confirm the hypothesis that L-type and/or R-type calcium channels play a more crucial role in pathology of visceral pain in animals.

A moderate degree of mechanical duodenal distension (DD20 and DD30) in sheep reduced the frequency of forestomach and abomasum motor activity by 45 and 52%, respectively [30], whereas, strong distension (DD40 and DD80) provoked the total contraction inhibition in conscious animals accompanied by the acute visceral pain [21, 33]. There is a direct relationship between the viscero-visceral reflex and visceral pain [34]. Visceral pain is a general sign involved in many gastro-duodenal and gastro-colonic disorders, such as colic, inflammatory

processes, and other diseases. These symptoms are accompanied by stimulation of the HPA (neuropeptides, hormones, e.g., cortisol) and SAS (catecholamine, 5-HT, neuropeptides) axes and the exacerbation of motivational and motor CNS structures (limbic system) involved in many quinine, neuropeptides, and necrohormones release useful in alarm reactions and defense of animals.

I.C.V. application of VGCCIs in 10 min premedication prevented nocifensive signs of behavior, clinical symptoms, increase plasma cortisol and catecholamine concentration in periphery, and perhaps in CNS structures, as well. The molecular mechanisms of these processes are the result of the L-type voltage-gated calcium channel inhibitors blockage of specific Ca^{2+} receptors by the drugs tested. Calcium channels receptor blockage by VGCC inhibitors attenuates visceral pain by inhibiting nocifensive neurohormone/neurotransmitters release in CNS and in peripheral nervous system, due to the fact that Ca^{2+} ions cannot bind to their specific receptor for depolarization of presynaptic neuronal membrane and promote the release of nocifensive substances.

4.1. Other types of calcium channel blockers used in the treatment of pain

Voltage-gated calcium channels are made with subunit α_1 which forms a channel pore and subunit $\alpha_{2\delta}$, which facilitates movement to the membrane surface [35]. There are ten different $\alpha_{1\delta}$ subtypes and four $\alpha_{2\delta}$. $\alpha_{2\delta1}$ and $\alpha_{2\delta2}$ subtypes bind gabapentin and pregabalin. Subtype 1 exists principally in the intermediate matrix (dentate gyrus, insula, cortex, and amygdala) [36].

Subtypes $\alpha_{2\delta1}$ exhibit expression also in dorsal root ganglia, spinal cord and in the small intestine smooth muscle, together with N-type calcium channels [36]. Subtype 2 is found in the periaqueductal gray matter, spinal cord and as diffused all over CNS, but not in the colon or duodenal smooth muscle [37]. Pregabalin and gabapentin bind with subtypes $\alpha_{2\delta}$ in the cytoplasm and prevent calcium channels expression on the plasmatic membranes [38]. Preventing the binding and expression blocks calcium conductance and in consequence substance P (SP), calcitonin gene related peptide (CGRP) and glutamate cannot be released from primary afferent neurons [35, 39]. Prevention of nocifensive neurotrasmitters release by gabapentine and pregabaline occurs only during pathological processes, in which calcium channels are up-regulated and activated [40]. Both pregabaline and gabapentine are central analgesics [39]. Gabapentinoids inhibited visceral hypersensitivity in the experimental animals, as well as irritable bowel syndrome in humans [41]. Smalls doses of gabapentine administered with morphine inhibit *i.p.* acid injection induced writhing syndrome in rats, which were ineffective when the drugs were applied separately.

Gabapentenoids not only inhibit central nociceptive transmission, but also enhance the intestine susceptibility to distension, possibly by blockage of $\alpha_{2\delta}$ subtypes in the smooth muscle [42, 43].

Other calcium channels can be also involved in the development of visceral hypersensitivity. Stimulation of T-type calcium channels, subtype $Ca_v3.2$ on the primary signaling visceral afferents was associated with symptoms similar to the irritable bowel syndrome in the animal model. Behavioral symptoms resolved after the application of T-type calcium channels

inhibitor [44]. Afferent transduction from mesentery in the experimental intestinal ischaemia was blocked by nifedipine, an L-type calcium channels inhibitor [45].

5. Conclusion

The results of this investigation indicates, that VGCCIs can be applied effectively in visceral pain modulation of animals and could be paid to the use of this kind of medicine, perhaps in human pain treatment as well.

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