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Effect of amiloride and bumetanide on transepithelial ion transport in isolated rabbit cecal and colonic wall

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction: The aim of the study was to compare the effects of amiloride and bumetanide on the baseline transepithelial electrical potential difference (PD) and changes in PD during mechanical stimulation (dPD) in isolated cecal and colonic wall of rabbits.

Materials/Methods: The experiments were performed with a modified Ussing chamber system. Isolated tissue specimens were incubated in Ringer's solution, in amiloride and/or bumetanide, or in dimethyl sulfoxide (DMSO).

Results: Under control conditions, i.e. when all the experimental fluids were Ringer's solution, the PD and R values of the rabbit cecum and colon were similar, while during mechanical stimulation, dPD of the colon was twice as high as that of the cecum. Addition of amiloride and/or bumetanide to all experimental fluids diminished the electrophysiological parameters of both tissues. DMSO added to all experimental fluids significantly diminished the values of the electrophysiological parameters of the cecum. Addition of amiloride to the stimulation fluid only diminished the PD and dPD values in the colon, whereas addition of bumetanide to the stimulation fluid only diminished the PD and dPD values in the cecum. It was found that the PD and dPD values of the rabbit cecum depend primarily on chloride ion transport, while those of the colon depend on sodium ion transport.

Key words: **colon wall • cecum wall • transepithelial electrical potential difference • electrogenic ion transport • ussing methods**

Abbreviations: **AMI** – amiloride; **AMI+BUME** – mixture of amiloride and bumetanide; **BUME** – bumetanide; **CGRP** – calcitonin gene-related peptide; **DMSO** – dimethyl sulfoxid; **dPD** – difference between the maximum stimulated value and the control value of PD; **NANC** – non-adrenergic, non-cholinergic; **NKA** – neurokinin A; **PD** – transepithelial electrical potential difference; **R** – transepithelial electrical resistance; **RH** – Ringer's solution; **SP** – substance P

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Introduction

The physiological and pathophysiological aspects of the transepithelial ion transport processes in the gastrointestinal tract wall have been widely studied [8,10,12,13,16–18, 25,27,31,35]. The electrophysiological equivalent of transepithelial ion currents is transepithelial electrical potential differences. It has been shown that mechanical stimulation, such as distension of the intestinal wall or stroking the surface, causes changes in electrogenic ion transport.

The gastrointestinal tract is covered with mucus lining [1,2,9,19,20]. It is supposed that electrogenic ion currents interact with the mucus lining and influence the contents of the colon.

Transepithelial ion transport processes can be regulated by intrinsic or extrinsic elements of the nervous system. Literature reports state that C-fiber sensory ending stimulation results in the release of NANC system neurotransmitters (e.g. NKA, SP, CGRP) which modify ion transport through the epithelia [6,26,28].

The aim of this study was to evaluate the transepithelial ion currents forming the baseline transepithelial electrical potential difference (PD) and changes in transepithelial electrical potential difference during mechanical stimulation (dPD) in the rabbit cecal and colonic epithelium.

Materials and Methods

The experiment was carried out on 125 and 202 specimens of isolated cecum and colon wall, respectively, from sixty 12-month-old rabbits. For the experiments, rabbits (with free access to water and food prior to the study) of both sexes and weighing between 3.5-4.0 kg were used. The experiment had been previously approved by the local Universities Committee for Ethical Animal Experiments.

Before each experiment, the feces of the rabbit underwent coproscopic examination for parasites.

The electrophysiological parameters measured by Ussing methods were the transepithelial electrical potential difference (PD), changes in the transepithelial electrical potential difference during mechanical stimulation (dPD), and transepithelial electrical resistance (R) [21]. PD was established when the compensation current intensity of the external battery was I = ±0 mA. Transepithelial electrical resistance was measured using electric impulses of I = ±10 mA, which were followed by respective voltage change measurements, whence R was calculated by Ohm's law. R was determined before and after stimulation.

A modified Ussing system was used in the experiments. The modification of the conventional Ussing chamber consisted in placing the tissue horizontally. To one half of the chamber a nozzle was mounted connected to a peristaltic pump. The jet of stimulation fluid from the peristaltic pump was a gentle mechanical stimulus applied to the mucosal surface of the tissue.

The halves of the chamber were connected by two pairs of agar bridges, mounted on both sides of the tissue, to silver/silver-chloride electrodes. The electrodes were then linked to a voltage/current clamp apparatus, EVC4000 (WPI, USA) and MP 100 (Biopac, USA) or a BD 111 recorder (Kipp&Zonen, The Netherlands). The first pair of electrodes was used for measuring the transepithelial electrical potential difference and the second for passing through electrical impulses.

The rabbits were killed by carbon dioxide asphyxiation. After incision of the abdominal wall, cecum and distal colon specimens about 10 cm long were gently excised, rinsed of chyme, cut longitudinally, and divided into pieces of about 2.5 cm^2. After 1 hour of incubation, each specimen was mounted in Ussing chambers filled with bathing fluid. After 15 min the mucosal surfaces of the tissues were stimulated with the stimulating fluid from the nozzle. The nozzle, 1.5 mm in diameter, was set 12 mm from the intestinal surface. The standard stimulus lasted 15 s and consisted of 7–8 fluid discharges of a total volume of 1.8 ml.

Each measurement was followed by an experiment with a synthetic cellophane membrane. The current-clamp measuring mode was applied for this test, with the current set at a level of ±80 μA, which allowed obtaining a PD of ±2 mV on the cellophane membrane. Next, the cellophane was stimulated with all the stimulating fluids applied in the experiment. This procedure was regarded as the "blind" test.

The following fluids were used in the experiments (concentrations in mM): stock solution: Ringer's solution containing Na^+ (147.2), K^+ (4.0), Ca^{2+} (4.4), Cl^- (155.6), and HEPES (10.0), buffered to pH 7.4; amiloride (0.01) dissolved in and diluted with Ringer's solution; bumetanide (0.01) dissolved in dimethyl sulfoxide (DMSO, final concentration 0.1%) and diluted with RH; and – DMSO (0.01%) dissolved in and diluted with RH.

All values are expressed as mean ±S.D. The Student's t-test was used to determine the statistical significance of differences between means. The value of $p<0.05$ was considered as the significance level. The analyses were performed using the "Statgraphics" software package.

Results

The electrophysiological parameters of rabbit cecum and rectum under different experimental conditions are presented in Tables 1 and 2.

Baseline transepithelial electrical potential difference

Under control conditions, i.e. when Ringer's solution was the incubation and bathing fluid, the PD and R values of cecum were –3.3±0.4 mV and 3.0+0.4 $k\Omega \times cm^2$, respectively. The PD value of the colon was similar, whereas R values of the distal colon were about 21% higher than in the cecum. Incubation of the tissue in RH with addition of amiloride decreased the PD values in cecum and distal colon by about 24% and 50%, respectively. There was no effect on the R value. After two consecutive 30-min. incubation periods, first in Ringer solution with amiloride (0.01 mM) and then without the drug, stimulation of the tissue with RH diminished the PD and R values in the ce-

Table 1. Baseline (PD), changes in transepithelial potential difference (dPD) and tissue resistance of isolated cecum and distal colon wall before and after inhibition of Na^+ and Cl^- ion transport pathways.

Experimental group	Experimental organ	PD (mV)	R (kΩ × cm²)	dPD (mV)
RH	Cecum n=20	–3.3±0.4	3.0±0.4	–1.2±0.3
	Distal colon n=38	–3.2±1.8	3.8±1.0	–2.2±1.0
AMI	Cecum n=20	–2.5±0.1*	2.7±0.4	–0.6±0.0*
	Distal colon n=32	–1.6±0.8*	3.5±1.3	–0.8±0.3*
RH after AMI	Cecum n=14	–1.4±0.2*	2.1±0.3*	–1.0±0.2
	Distal colon n=28	–8.5±0.8*	4.6±1.2	–4.9±1.7*
BUME	Cecum n=20	–0.9±0.2*	1.1±0.5*	–0.2±0.1*
	Distal colon n=30	–2.3±0.9	3.0±0.8	–1.5±0.4*
AMI+BUME	Cecum n=20	–0.7±0.2*	2.6±0.3	–0.3±0.1*
	Distal colon n=20	–0.5±0.3*	2.9±1.0	–0.3±0.1*
DMSO	Cecum n=11	–1.4±0.3*	0.9±0.2*	–0.3±0.0*
	Distal colon n=26	–4.1±1.1	3.4±1.0	–1.7±0.8

The mean ± S.D. value are given; n – number of experiments; PD – baseline transepithelial electrical potential difference; dPD – the difference between the maximum stimulated value and the control value of PD; R – transepithelial electrical resistance.
The tissue was investigated in the following solutions (concentrations given in mM in parenthesis): RH – Ringer's solution without additions, AMI – Ringer's solution with addition of amiloride (0.01); BUME – Ringer's solution with addition of bumetanide (0.01); DMSO – Ringer's solution with addition of dimethyl sulfoxide (0.1%); AMI + BUME – Ringer's solution with addition of amiloride (0.01) and bumetanide (0.01); RH after AMI – two consecutive 30-min. incubation periods, first in Ringer's solution with amiloride (0.01) and then without.
* statistically significant difference compared with RH group ($p<.05$).

Table 2. Effect of amiloride and bumetanide on PD and dPD of rabbit cecum and rectum without preincubation.

Experimental group	RH dPD (mV)	AMI dPD (Mv)	BUME dPD (mV)	DMSO dPD (mV)
Cecum n=20	–1.2±0.3	–0.3±0.0*	–0.4±0.1*	–0.5±0.1*
Distal colon n=28	–2.2±1.0	+2.8±1.4*	–1.5±0.4*	–2.1±1.3

The mean ± S.D. value are given; n – number of experiments; dPD – the difference between the maximum stimulated value and the control value of PD. The incubation and bathing fluids were Ringer's solution; the stimulation fluids were as follows (concentrations given in mM in parenthesis): RH – Ringer's solution; DMSO – Ringer's solution with dimethyl sulfoxide (0.1%); BUME – Ringer's solution with bumetanide (0.01); AMI 100 – Ringer's solutions with amiloride (0.01).
* statistically significant difference compared with RH group ($p<.05$).

cum by about 44% and 22%, respectively, whereas the PD value in the colon increased five-fold and R was unchanged compared with AMI incubation (Table 1). Incubation of the tissue in Ringer's solution with the addition of bumetanide decreased the PD values in cecum and distal colon by about 73% and 28%, respectively. With an inhibited chloride ion transport pathway, decreased R values were observed in the cecum and distal colon, by about 63% and 20%, respectively. Application of amiloride and bumetanide simultaneously to the incubation fluid decreased the PD values in the cecum and distal colon by about 79% and 84%. With an inhibited sodium and chloride ion transport pathway, decreasing R values were observed in the cecum and distal colon, by about 13% and 24%. After incubation in Ringer's solution with the addition of DMSO, the PD and R values of the cecum were decreased by about 58% about 78% compared with the control incubation (Table 1). In contrast, the PD value of the distal colon increased by about 22%, while R decreased by about 10%.

Changes in transepithelial electrical potential difference during mechanical stimulation

Mechanical stimulation by gentle washing of the mucosal surface of the tissue caused a transient increase in the PD value, observed as hyperpolarization (dPD) in the cecum and distal colon (Table 1, Figure 1).

Incubation of the tissue in RH with addition of amiloride decreased the dPD values in the cecum and distal colon by about 50% and 64%, respectively (Table 1, Figure 2).

After two consecutive 30-min. incubation periods, first in Ringer's solution with amiloride and then without the drug,

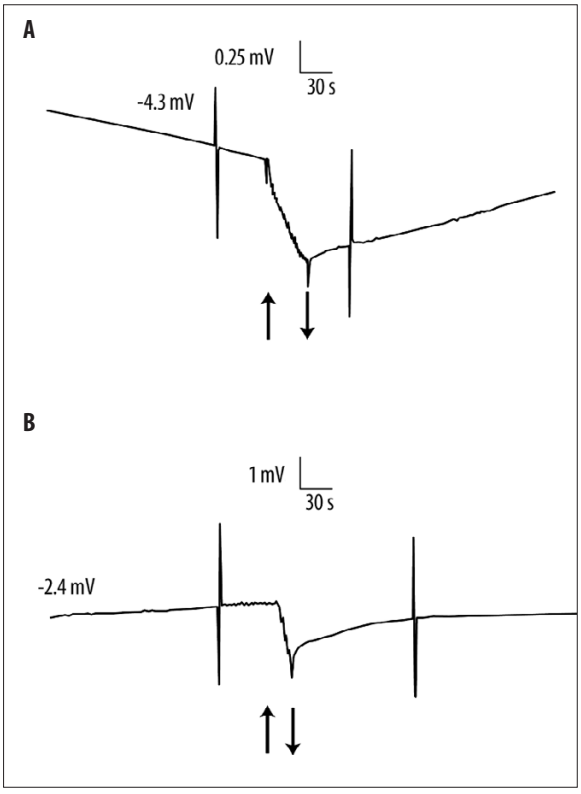

Figure 1. Typical record of hyperpolarization after mechanical stimulation of isolated rabbit cecum (A) and distal colon (B) by jet rinsing from peristaltic pump. The tissue was incubated and bathed in Ringer's solution. The arrows denote the beginning and end of the stimulus.

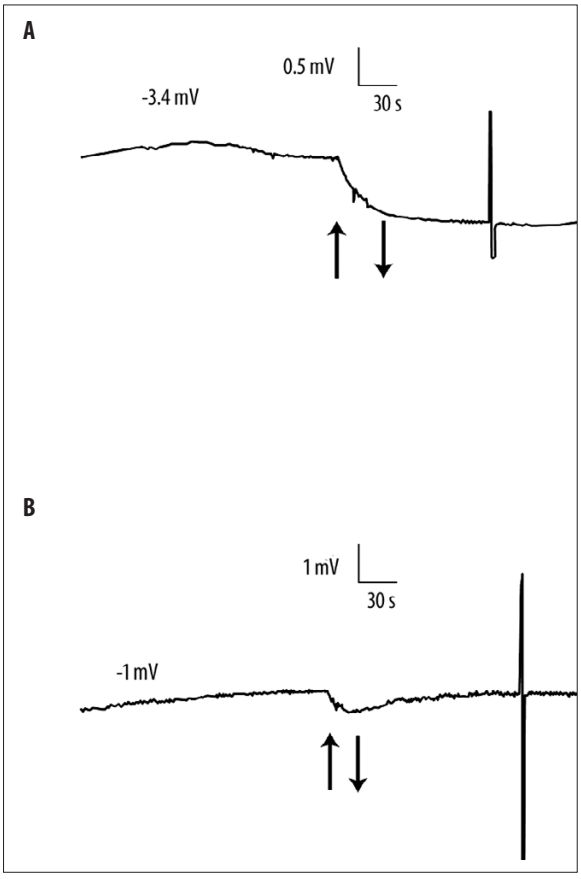

Figure 2. Changes in transepithelial electrical potential difference of cecum (A) and distal colon (B) after mechanical stimulation. The tissue had been incubated in Ringer's solution with an addition of amiloride. The moments of stimulus are marked with arrows.

stimulation of the tissue with RH diminished the dPD value in the cecum by about 40%, whereas in the colon, dPD was augmented by about 25% compared with AMI incubation (Table 1). Incubation of the tissue in Ringer's solution with addition of bumetanide decreased the dPD values in the cecum and distal colon by about 83% and 32%, respectively (Table 1, Figure 3).

Application of amiloride and bumetanide simultaneously to the incubation fluid decreased the dPD values in the cecum and distal colon by about 75% and 86%, respectively. After incubation in Ringer's solution with DMSO, the dPD values of the cecum and colon decreased by about 75% and 23%, respectively; in some experiments an increase in the dPD value of the colon was observed (Table 1).

Direct effects of amiloride and bumetanide on PD and dPD values

Adding ion transport inhibitors to the stimulation fluid without preincubation after incubating the tissues in RH caused different effects in the cecum and colon. Amiloride added to the stimulation fluid only diminished the hyperpolarization of the cecum by about 75%, while it caused a depolarization effect in the colon (the PD value was less negative) (Table 2). Bumetanide added to the stimulation fluid only diminished dPD of the cecum and distal colon by about 67% and 30, respectively (Table 2). Application of DMSO to the stimulation fluid diminished the cecal dPD value by about 77% and did not influence the value of the response to mechanical stimulation (Table 2).

Discussion

The results presented in this study revealed that various ion transport pathways form the PD and baseline transepithelial electrical potential difference (PD) and changes in the transepithelial electrical potential difference (dPD) in the rabbit cecum and distal colon.

The transepithelial electrical potential difference is an electrophysiological result of transepithelial sodium and chloride ion transport pathways through transporters located in the apical and basolateral membranes of epithelial cells [7,14,15,21–24,32–34,36]. These processes lead to segregation of electrical charges. Negative charges gather on the mucosal layer covering the rabbit large intestine, while the positive charges gather on the serosal side.

In our previous reports we described PD and dPD [21–24, 32–34]. PD is a parameter dependent on ion transport processes regulated over long time spans. dPD is a short-lived parameter associated with mechanical stimulation. dPD was mainly observed as transient hyperpolarization resulting from enhanced chloride secretion and sodium reabsorption.

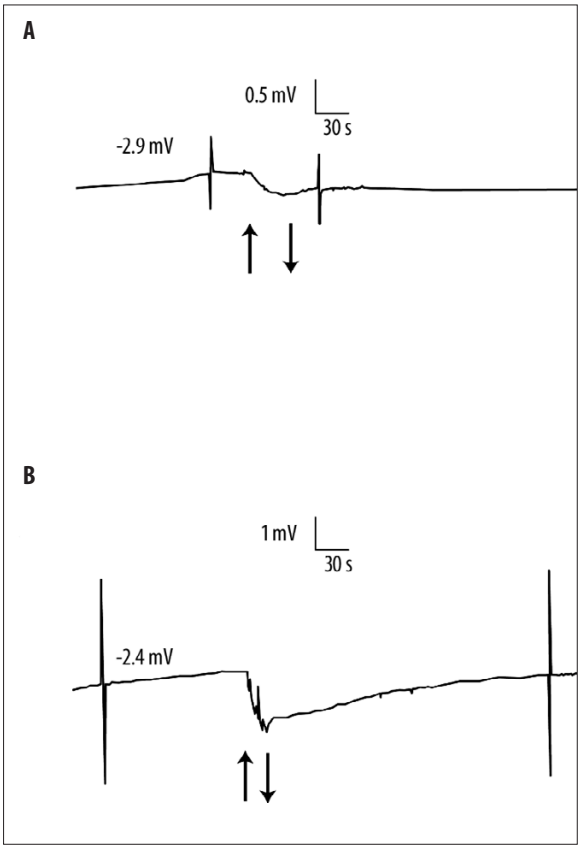

Figure 3. Effect of bumetanide on transepithelial electrical potential difference of isolated cecum (A) and distal colon (B) walls *in vitro* after mechanical stimulation. The tissue had been incubated and washed with Ringer's solution with an addition of bumetanide.

Experimental models

This study was exclusively devoted to comparing which ion currents form PD and dPD in the rabbit cecum and colon. In most studies, a voltage clamp was set to estimate ion transport processes [21–24,32–34]. In this study the experiments were performed under the open-circuit condition so that the function of voltage-gated channels would be fully preserved. Amiloride and bumetanide were added separately or simultaneously to the experimental fluids to determine whether PD and dPD depend on chloride, sodium, or other ion currents. In the experiments, ion transport inhibitors were added to all the experimental fluids or to the stimulation fluid only. In the latter procedure, ion transport inhibitors, added without preincubation, evoked an "immediate" effect on the PD and dPD values. Both modes of application confirmed which ion transport pathways (chloride, sodium, or other) participate in the formation of PD and dPD. In the experiments, concentrations of amiloride and bumetanide (0.01 mM) were used that were comparable to those of other studies [3]. The concentration of 0.01 mM of AMI or BUME was adequate to evoke the reaction of diminution of the specific transport pathway.

The stimulation applied in this study was the pulsatory movement of fluid across the surface of the tissue, which stimulated epithelial surface receptors.

Baseline transepithelial electrical potential difference

It was demonstrated that PD values in the cecum and colon are similar (Table 1) R showed significant differences. In order to identify the ion transport pathways which participate in forming the PD value in the rabbit large intestines, two ion transport inhibitors were applied, i.e. amiloride for sodium ions and bumetanide for chloride ions.

Amiloride, a known selective blocker of epithelial sodium channels, blocks the channels in a quick and reversible way [3,5,22–24,29,30,32–34]. AMI concentrations of 0.1 to 1 μmol/l [5] block sodium channels in the mammalian large intestinal epithelium.

Application of AMI can inhibit the sodium ion absorption and allows chloride ion secretion to prevail. Application of amiloride to the incubation fluid resulted in a slightly reduced PD of the rabbit cecum and a significantly reduced PD of the distal colon (Table 1). This showed that sodium ion absorption is more responsible for the PD in the distal colon than in the cecum. AMI had no significant effect on transepithelial resistance, both in the cecum and colon.

Bumetanide, a transepithelial chloride ion transport blocker, was another inhibitor applied in the study. BUME blocks the basolateral $Na^+K^+2Cl^-$ contransport mechanism [21–23]. With the presence of BUME in the incubation and stimulation fluids, the values of PD, as well as of the dPD response, depend entirely on sodium ion transport. Application of ion transport inhibitors to experimental fluids can be referred to as pharmacological isolation of the exact ion current. Application of AMI or BUME to the incubation of the rabbit cecum and distal colon did not entirely block the PD value (Table 1). It may be presumed, therefore, that, interchangeably, either chloride or sodium ions form the PD value.

In contrast, bumetanide applied in the incubation reduced the values of PD, R, and dPD of the cecum (Table 2). After incubation with BUME, the electrophysiological parameters of the rectum were also found to have dropped, but the reduction was not as great as in the case of the cecum.

Changes in transepithelial electrical potential difference during mechanical stimulation

The mechanical stimulation applied in the study as a gentle washing of the mucosal surface of both the cecum and colon caused transient hyperpolarization: the PD value was more negative (Figure 1). The dPD value of the rabbit distal colon was twice as high as that of the cecum (Table 1). Application of AMI to the incubation and stimulation fluids significantly diminished the dPD value of the rabbit's distal colon and cecum (Table 1, Figure 2). The hyperpolarization after gentle washing of the mucosal surface of the distal colon depended on sodium ion absorption (Figure 2B). In contrast, application of BUME to the incubation and stimulation fluids significantly diminished the dPD value of rabbit cecum (Table 1, Figure 3A).

The hyperpolarization after gentle washing of the mucosal surface of the cecum depended on chloride ion secretion. Application of AMI and BUME to all the experimental

fluids revealed that the reaction of hyperpolarization after gentle washing of the mucosal surface of the tissue is preserved by other ion transport processes, even after complete inhibition of sodium and chloride currents. The procedure with two incubation periods, first in the presence of AMI and then in Ringer's solution, was used to evaluate the reversibility of the reactions blocked by amiloride. This procedure also revealed that transepithelial ion transport processes, especially in the colon, in a physiological state are not maximally expressed.

Direct effects of amiloride and bumetanide on PD and dPD values

Some differences were obtained after adding ion transport inhibitors without preincubation. AMI added to the stimulation fluid only diminished the dPD value by about 75% in the cecum and caused a depolarization response in the distal colon (Table 2). BUME added to the stimulation fluid significantly diminished the hyperpolarization response in the cecum only, and slightly in the distal colon (Table 2). It has demonstrated that sodium currents are the main ions responsible for colonic PD, while both transport channels for sodium and chloride ions take part in the cecal response.

Regulation of ion transport processes

Results of our studies, as other reports, confirmed that neuron endings sensitive to mechanical stimuli located in the intestinal epithelium take part in the response to gentle washing of the mucosal surface [1]. A hypothetical mechanism can be that pulsatory washing of tissue triggers the release of the NANC system neuropeptides from the sensory endings, which modify sodium and chloride ion transport in the large intestinal epithelium.

The main subpopulation of sensory neurons located in the epithelia are C-fibers, which play two basic functions, i.e. sensory and motoric. It has been demonstrated that excited sensory neurons not only send afferent impulses, but also release the NANC system neuropeptides [4,6,26,28]. A number of substances are able to excite the C-fibers. These include inflammatory mediators (such as histamine, prostaglandins PGF-2 alpha, PGE-2, and PGI-2, as well as bradykinin), irritating agents (capsaicin, sulfur dioxide, nicotine, citric acid, and lobeline), as well as osmotically active substances (e.g. distilled water) [6,11,24,26,28,37]. Dimethyl sulfoxide (DMSO, a compound commonly used in biochemical analyses and cellular biology) is a medium that also affects C-fiber endings [24,37].

In the presented studies, DMSO was applied to all experimental fluids or to the stimulation fluid only. Application of DMSO to the stimulation fluid resulted in a diminution of the hyperpolarization response in the cecum and did not affect or augmented the dPD value of the colon (Table 2). A similar effect on PD and dPD values was found when DMSO was added to all experimental fluids (Table 1). It can be assumed that DMSO inhibited neuropeptide release from sensory neurons during mechanical stimulation in the rabbit cecum, whereas it did not effect or augmented neuropeptide release in the colon.

On the base of these results it can be concluded that the PD and dPD values of the rabbit cecum depend primarily on chloride ion transport, whereas those in the colon depend on sodium ion transport. This can result from the different functions of the cecum and colon in accumulating and propelling their contents. It can be assumed that gentle washing of the mucosal surface caused stimulation of superficial neurons endings because DMSO modified electrogenic ion transport in both the rabbit cecum and distal colon epithelium.

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