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EFFECTS OF AMILORIDE AND BUMETANIDE ON ION TRANSPORT IN THE CAECUM OF RABBIT

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Effect of selective blockers of sodium and chloride ion transport (amiloride and bumetanide) on transepithelial electrical potential (PD), transepithelial electrical potential difference (dPD), and electrical resistance of the tissue (R) of isolated fragment of the rabbit's caecum were determined using electrophysiological methods designed for measuring ionic currents occurring in epithelial tissues.

A modified Ussing apparatus enabling application of mechanical and chemical stimuli on the isolated tissue was used in the experiment.

It was demonstrated that amiloride used for incubation of the caecum fragments lowered by some 24% the value of PD and by 50% the value of dPD. Incubation of the caecum fragments with bumetanide resulted in a decrease in the PD value by 73% and in the value of dPD by some 83%. The results obtained with the tissue incubated in Ringer solution with addition of both compounds were comparable with those observed for the tissue incubated with bumetanide.

As can be concluded from the above-mentioned experiments, both ion transport pathways contribute jointly to the induction of PD in the epithelium of the rabbits caecum.

Key words: *amiloride, bumetanide, caecum, ion transport, rabbit*

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Abbreviations: AMI – *amiloride,* BUME – *bumetanide,* dPD – *the difference between maximal stimulated value and control value of PD,* NANC – *non-adrenergic, non-cholinergic,* PD – *transepithelial potential difference (mV),* R – *transepithelial electrical resistance (ohm x cm^2)*

INTRODUCTION

Processes of absorption of sodium ions and secretion of chloride ions play an important role in maintaining functions of excretory epithelia. They coordinate mucous-microvilli clearance and ionic composition of liquid lining of the epithelium of the respiratory tracts, and they influence viability of sperm cells and implantation of embryo in the uterine wall [3, 8, 9, 14, 29, 32–34, 36]. Processes of absorption and secretion of fluids and electrolytes play also an important role in the large intestine of mammals, regulating, among other things, also mucus layers covering the epithelium of the large intestine [13, 16, 21–23, 35]. The mucus secreted by the goblet cells not only protects the wall of the large intestine against mechanical damage and bacterial toxins, but it also facilitates formation of fecal masses and their intestinal passage. Irritation of the large intestine results in diarrhoea because of increased secretion of water and mucus [12, 31].

In physiological sense, the functional state of the large intestine is expressed by the value of transepithelial electrical potential (PD) induced mainly as a result of sodium and chloride ion transport [21, 22]. Many studies aiming to identify the ion transport pathway (pathways) utilized amiloride (AMI) and bumetanide (BUME). AMI blocks sodium channels in a quick and reversible way, through limitation of the channel conductivity [1, 2, 5, 7, 8, 11, 15, 30].

According to Frizzell's hypothesis, the epithelial sodium channels occurring in the large intestine of rabbit are blocked by AMI concentrations exceeding 1 μmol/l [10]. Using AMI as a selective sodium channel blocker, we can inhibit the absorption phase of sodium ions and obtain a predominance of chloride ion secretion [5, 33]. On the other hand, BUME is a commonly used inhibitor of transepithelial transport of chloride ions through blockage of basolateral mechanism of $Na^+K^+2Cl^-$ cotransport [25, 28].

The aim of this work was to study the action of mechanical and chemical stimuli on the PD, transepithelial electrical potential difference (dPD), and the electrical resistance of the tissue (R) in the epithelium of an isolated rabbit caecum wall before- and after pharmacological modification of ion transport pathways by selective transport blockers AMI and BUME.

MATERIALS and METHODS

The experiments were carried out on 80 fragments of caecum collected from 20 not outbred rabbits of both sexes, provided by the Animal Experimental Unit of the Pomeranian University in Szczecin. They consisted of measuring PD, dPD, and R of the fragments of an isolated caecum wall placed in an Ussing apparatus [20]. The readings were tested by EVC4000 device (manufactured by WPI, USA) and BD recorder 111 (Kipp & Zonnen, the Netherlands) connected to the Ussing apparatus by ClAg electrodes and agar bridges filled with KCl solution. Electrical stability of the measuring system was tested applying solutions on a synthetic cellophane membrane placed in the Ussing apparatus (blind sample).

The rabbits were killed by suffocation with carbon dioxide and the tissues were immediately sampled. The caecum was dissected and its content was removed by gentle rinsing. Subsequently, it was placed in Ringer solution at 36°C, stripped of connective tissue, cut open longitudinally, and divided into pieces. Tissue samples prepared in such way were incubated and were placed one by one in the Ussing apparatus. The mechanical stimulus was a stream of the fluid from the chamber of the Ussing apparatus flushing the mucous surface of the caecum. The stream was ejected within 15 s from a nozzle, 1.5 mm in diameter, situated 12 mm from the surface of the studied tissue.

The media used for incubation, filling up chambers of Ussing apparatus, and the stimulation during the experiment were:

— Ringer solution (its ionic composition is given in mmol/l): Na^+ 147.2, K^+ 4.0, Ca^+ 4.4, Cl^- 155.6, HEPES 10.0,
— Ringer solution supplemented with AMI (0.1 mmol/l),
— Ringer solution supplemented with BUME (0.1 mmol/l),
— Ringer solution supplemented with AMI and BUME (all supplied by Sigma Chemical Co.).

Statistical hypotheses were verified by chi square test for assessment of the qualitative data and Student's *t*-test for quantitative data ($p < 0.05$). The data were computed using "Statgraphics" computer software.

RESULTS

Table 1 shows the effect of AMI and/or BUME on the value of PD, dPD, and R of the studied tissue, following a mechanical stimulation. Isolated fragment of the rabbit caecum, incubated in Ringer solution always reacted with transient hyperpolarization to mechanical stimulation. When the stimulation was over, the electrical potential returned to the initial value observed before the mechanical stimulation (Figs. 1a, 2a).

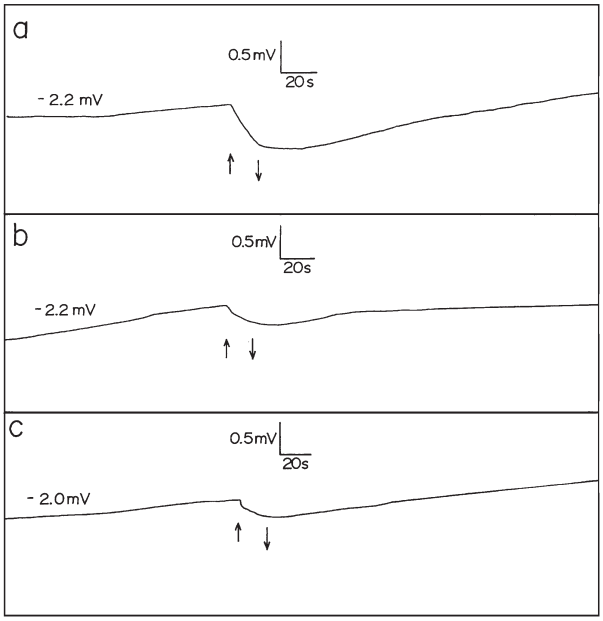

Fig. 1. Change in electrical potential of the isolated rabbit caecum caused by mechanical and chemical stimuli. Caecum fragments were incubated in Ringer solution. The stimulation consisted in rinsing the mucous surface for 15 s with Ringer solution (a), Ringer solution with addition of amiloride (b), and Ringer solution with addition of bumetanide (c)

In the course of the tissue incubation in the presence of AMI, the value of PD decreased by some 24% and the value of R by 60% compared to control incubation (in Ringer solution). Under such experimental conditions, the reaction to the mechanical stimulation was lower by about 50% compared to the control stimulation. After the stimulation ended, the value of PD did not return to the initial value, observed before the stimulus application (Fig. 2b).

Incubation of rabbit caecum fragments with addition of BUME caused lowering of PD value by some 73% and the value of R by about 20% in relation to control incubation. Comparison of PD and R values in the groups treated with AMI and BUME, indicated that the value of PD in the group of tissues incubated with BUME was by some 64% lower, than in the group of tissues incubated with AMI. On the other hand, the value of R in BUME group was by some 50% higher than in AMI group.

Incubation of rabbit caecum fragments in the presence of BUME decreased the reaction to mechanical stimulation by some 83% compared to control stimulation (Fig. 2c). Comparison of this value with the reaction of the tissue incubated with AMI to mechanical stimulation demonstrated that in AMI group this value was by 67% higher, then in BUME group.

Joint application of both selective transport blockers caused PD lowering by some 79% and R drop by some 40%, compared to control value.

Table 1. Effect of incubation of rabbit large intestine with ion transport inhibitors on electrophysiological parameters of rabbit caecum

Incubation coditions (n)	PD (mV)	R (kΩ*cm^2)	Mechanical stimulation	
			PD (mV)	dPD (mV)
RH n = 20	–3.3 ± 0.4	0.1 ± 0.0	–4.7 ± 0.4	–1.2 ± 0.3
AMI n = 20	–2.5 ± 0.1*	0.04 ± 0.0*	–3.9 ± 0.3	–0.6 ± 0.0*
BUME n = 20	–0.9 ± 0.2*	0.08 ± 0.0	–1.8 ± 0.2	–0.2 ± 0.1*
AMI + BUME n = 20	–0.7 ± 0.2*	0.06 ± 0.0*	–2.0 ± 0.2	–0.3 ± 0.1*

The values represent the mean ± SE; n – number of studied fragments of rabbit caecum; RH – Ringer solution; AMI – Ringer solution with amiloride; BUME – Ringer solution with bumetanide dissolved in DMSO; AMI+BUME – Ringer solution with addition of amiloride and bumetanide; * significantly different in relation to the values in RH group ($p < 0.05$)

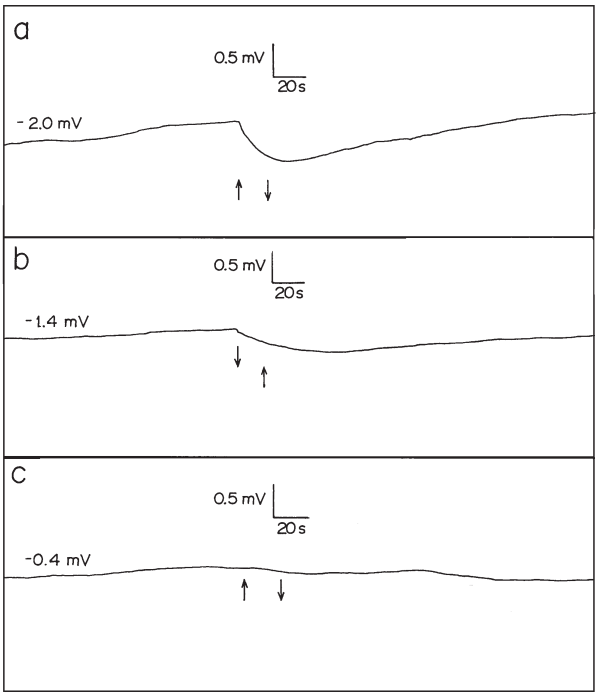

Fig. 2. Effect of inhibitors of transepithelial transport of sodium and chloride ions on the changes in electrical potential of the isolated rabbit caecum following the action of mechanical stimuli. The stimulation consisted in rinsing the tissue for 15 s with the liquid currently present in the Ussing apparatus. Incubation conditions: Ringer solution (a), amiloride (b), bumetanide (c). The arrow marks the application of mechanical stimulus

Table 2. Effect of mechanical and chemical stimulation on electrophysiological parameters of the isolated wall of rabbit caecum

Incubation conditions (n)	AMI	BUME	AMI + BUME
	dPD (mV)	dPD (mV)	dPD (mV)
RH n = 20	–0.3 ± 0.0	–0.5 ± 0.1	–0.5 ± 0.1
AMI n = 20	–0.6 ± 0.2	–	–0.4 ± 0.1
BUME n = 20	–	–0.2 ± 0.0*	–0.2 ± 0.0*
AMI + BUME n = 20	–0.3 ± 0.1	–0.2 ± 0.0	–0.3 ± 0.0*

Explanation of abbreviations and symbols is given in Table 1

Table 3. Effect of mechanical stimulation, following chemical stimulation, on transepithelial electrical potential of the isolated wall of rabbit caecum

Incubation conditions (n)	RH after AMI	RH after BUME	RH after AMI + BUME
	dPD (mV)	dPD (mV)	dPD (mV)
RH n = 20	–0.7 ± 0.1	–0.4 ± 0.1*	–0.6 ± 0.1
AMI n = 20	–0.2 ± 0.1*	–	–0.5 ± 0.2
BUME n = 20	–	–0.2 ± 0.1	–0.3 ± 0.0
AMI + BUME n = 20	–0.4 ± 0.1	–0.6 ± 0.1	–0.2 ± 0.0*

Explanation of abbreviations and symbols is given in Table 1

This reaction to mechanical stimulation was comparable with the value obtained after incubation of the tissue with BUME.

Table 2 shows the values describing effect of AMI and/or BUME applied directly on the surface of the rabbit's caecum under different experimental conditions. After the incubation of the tissue in Ringer solution, the tissue was subjected to a chemical stimulus in a form of Ringer solution supplemented with AMI. It resulted in lowering by some 75% the reaction to stimulation. When the stimulation ended, the value of PD did not return to the initial level (Fig. 1b). Supplementation of the stimulating fluid with BUME and AMI in this group caused a decrease in dPD by some 58% compared to control stimulation (Fig. 1c). After tissue incubation with AMI, the application of AMI and BUME in the stimulation solution did not influence the hyperpolarization value. This reaction was comparable with the value after the mechanical stimulation. Similar situation occurred when the tissue was incubated with BUME and then with AMI and BUME.

The experiments also aimed to establish whether the effects of AMI and/or BUME under different experimental conditions were reversible. The data are shown in Table 3.

Application of AMI in the stimulating solution on the tissue incubated in Ringer solution caused a lowering of the value of mechanical stimulation-induced hyperpolarization by some 42%, compared to control stimulation. Similar reaction to mechanical stimulation was observed after the application of AMI and BUME.

After the incubation of the tissue with AMI, the stimulation with Ringer solution resulted in inhibiting the reaction by some 67% compared to mechanical stimulation of the tissue incubated with AMI. Incubation in a mixture containing both AMI and BUME and subsequent application of Ringer solution caused an increase in the reaction by some 60%, compared to the application of Ringer solution after AMI. The latter reaction was comparable with the value obtained after control stimulation (stimulation with Ringer solution with AMI).

Stimulation of the caecum fragments incubated in the presence of BUME with Ringer solution caused a reaction comparable to the stimulation with Ringer solution reinforced with BUME. After the tissue stimulation with the solution containing AMI and BUME, the reaction to the stimulation with Ringer solution alone did not change.

Stimulation of the tissue incubated in a mixture of AMI and BUME with Ringer solution triggered the reaction comparable to the value observed during mechanical stimulation. After the application of AMI, the stimulation with Ringer solution caused an increase in this reaction by some 50%, while after the application of BUME, it rose by some 67% compared to stimulation with RH solution after application of a mixture of AMI and BUME.

DISCUSSION

Isolated epithelial cells have been studied using electrophysiological *in vitro* methods since the 1950s [10] and the results obtained in such way have been fully confirmed by *in vivo* studies. The principal advantage of the *in vitro* experiments is the lack of regulatory actions (nervous, endocrine, and circulatory), which might interfere with interpretation of the results. For this reason, it is believed that the results obtained in that kind of studies reflect the ion transport occurring in the alimentary tract.

Epithelium, including the epithelium of rabbit caecum, may be in two states under physiological conditions: resting or active state. The resting state is characterized by stable PD, which is induced and modified by the processes of ion transport. On the other hand, the active state represents a hyperpolarization reaction, whose value is reflected by dPD. The dPD depends on the stimulation of sensory endings, neuropeptides released, and partial stimulation of the ion transport system [21, 22, 33, 34].

The dPD in the isolated caecum is an important physiological parameter, resulting from the segregation of electrical charges. Positive charges concentrate in the sub-mucous space of the caecum, while the negative ones gather in the mucous layer covering the intestine. The potential difference in rabbit caecum is induced by two transport pathways: the transepithelial pathway of chloride ion secretion and the transepithelial pathway of sodium ion resorption [3, 14, 32, 36].

It has been demonstrated in the present study that PD of an isolated rabbit caecum remains stable in the course of a several-hour experiment reaching the mean value of -3.3 ± 0.4 mV with the tissue resistance of 0.1 ± 0.0 kΩ*cm^2. Those values are comparable with parameters of ionic currents in the epithelium of rabbits trachea obtained by Tyrakowski et al., working with the same experimental set-up [33, 34]. In our earlier study on the flow of ionic currents in isolated fragments of the trachea and intestine of rabbit, it was demonstrated that physiological and pharmacological stimuli cause changes in ion transport in those tissues [21, 22, 33, 34].

Also a mechanical stimulus was used, which reflected the physiological situation [26]. It consisted in rinsing the mucous intestine surface with the solution used for incubation, which caused movement of mucus on the surface of the epithelium. In numerous electrophysiological studies on mucous membranes, it was demonstrated that such or similar actions stimulate sensory receptors and C-fibres in particular [6, 21, 22, 24, 26]. It has been demonstrated in this work that electrical potential is stable for long-time (up to several hours of *in vitro* observations), but may undergo short reversible (transient) changes in the form of hyperpolarization, during the action of mechanical stimuli (Tab. 2, Figs. 1a, 2a). Those changes indicate involvement of sensory endings (C-fibres) in this reaction which justifies the assumption that neuropeptides of the non-adrenergic, non-cholinergic (NANC) system (SP, NKA, CGRP and others) are released from sensory endings during the stimulation and they stimulate epithelial cells to change ion transport [13, 19, 23, 24, 27]. This problem is related mostly to the regulation of the liquid lining of the epithelium of respiratory tracts and its composition[4].

Regulation of liquid lining of the excretory epithelia, among others in respiratory tracts and alimentary tract, is associated with the transport of sodium and chloride ions [3, 9, 11, 14, 16–18, 29, 32–34, 36]. Sodium resorption decreases, while secretion of chlorides increases the liquid lining. It is possible that the changes in sodium resorption and chloride secretion have an adaptive value. In respiratory tracts, the increase in chloride secretion causes an increase in the liquid lining and the separation of excessive mucus lump from the epithelium, which enables its removal in the cough reaction [33]. The absence of the liquid lining triggers

secretion of chlorides, while the lining excess induces resorption of sodium ions [33, 34]. It is possible that the changes in ion transport after mechanical stimulation in an isolated rabbit caecum are similar to the processes occurring in respiratory tracts.

In order to identify the pathway (pathways) responsible for the changes in the electrical potential, two ion inhibitors were used: AMI and BUME. Those inhibitors were used in two different procedures. The immediate effect of those inhibitors were used to evaluate involvement of ion channels in inducing and changing PD. On the other hand, the other procedure consisting in incubation of tissue in the presence of an inhibitor of transport pathways, enabled to assess intraparietal reaction of control systems to the inhibition of functions of the ion channels. The immediate effect of AMI action consisted in increasing electrical potential by some 27%, on an average, and on lowering the hyperpolarization reaction by some 75% (Tab. 2, Fig. 1b). On the other hand, a prolonged AMI action consisting in incubation of the caecum in the presence of this compound, caused a slight decrease in PD, and a decrease in dPD during mechanical stimulation by some 50% (Tab. 1, Fig. 2b).

Reversibility of the reaction after the use of AMI depended on the duration of its action. Short action of AMI was partly reversible. Addition of Ringer solution after previous action of AMI on a caecum incubated in Ringer solution, caused an increase in the reaction by some 75% (Tabs. 2, 3). A prolonged action of AMI, however, was not reversible. Application of Ringer solution on preparations incubated with AMI not only did not increase the reaction, but even lowered it by some 67% compared to the mechanical stimulation (Tabs. 2, 3). It constitutes an evidence that under the condition of AMI-inhibited sodium ion transport, the changes in ion transport were not compensated by other transport pathways.

Prompt action of BUME slightly lowered PD, while dPD was recuced by some 58% (Tab. 2, Fig. 1c). Prolonged action of BUME caused PD decrease by some 73%, whereas dPD dropped by 83% (Tab. 1, Fig 2c). The reaction to BUME was reversible, irrespectively of the exposure time (Tab. 3).

Short-term presence of both inhibitors jointly resulted in dPD decrease by some 58% (Tab. 2). Incubation in the presence of AMI and BUME caused PD and dPD lowering by some 79% and 75%, respectively (Tab. 1).

The above-mentioned experimental data indicate that the reaction of the rabbit caecum to mechanical stimulation depends predominantly on the accessibility of apical ionic channels for chloride and sodium ions, while channels for other ions are less involved in this reaction.

CONCLUSIONS

1. PD of the isolated rabbit caecum wall is induced as a result of sodium ion absorption and chloride ion secretion, with more emphasis on chloride current.
2. Reversible mechanical stimulation-induced hyperpolarization of PD is dependant on stimulation of afferent nerve endings (sensory) and the release of neuropeptides of the NANC system, which stimulate the changes in the ion transport.

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