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Faculty of Pharmacy

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Differential reactivity of the longitudinal and circular muscle of the rat distal colon

Master thesis

Supervisors: Prof. Manuela Morato

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Porto and Hradec Králové 2016 Růžena Kubíčková

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Abstract (en)

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Supervisors: Prof. Manuela Morato

PharmDr. Ivan Vokřál, PhD.

Title of diploma thesis: Differential reactivity of the longitudinal and circular muscle of the rat distal colon

Physiological regulation of the function of the large intestine is neurohumoral. The neural part of this regulation implies the vegetative nervous system, which is crucial for the control of the gastrointestinal tract (GIT) motility. Concerning the humoral part of the regulation, recent studies showed that angiotensin II (Ang II) causes contraction of the colonic smooth muscle and, thus, can also influence the motility of the colon. However, there are no known studies that have described this process in detail. The aims of this work were (1) to compare the reactivity of the longitudinal and circular muscles of the rat distal colon to potassium chloride (KCl), acetylcholine (ACh) and Ang II, (2) to compare the observed results between male and female rats, and (3) to characterize the receptors mediating the response to Ang II. For purpose of the project, adult, 10-12 weeks old Wistar Han rats of both genders were used. Strips of the the rat distal colon were mounted in organ baths along their longitudinal or circular axis, and isometric responses were obtained. In both genders, the reactivity (g/g) of the circular muscle was higher than that of the longitudinal muscle. In the response to KCl, ACh and Ang II gender differences were observed only in the circular muscle. Concerning the response to Ang II, it seems that AT_1 receptors mediate contraction of the rat distal colon smooth muscle, while the AT_2 receptors mediate smooth muscle relaxation.

Abstrakt (čj)

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Katedra farmakologie a toxikologie

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Školitelé: Prof. Manuela Morato

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Název diplomové práce: Diferenciální reaktivita podélného a příčného svalu distálního tračníku potkana

Fyziologická funkce tlustého střeva je regulovaná neurohumorálně. Neurální část této regulace tvoří vegetativní nervový systém, který je zásadní pro kontrolu motility trávicího systému. Co se týče humorální části regulace, současné studie ukázaly, že Ang II způsobuje stah hladkého svalu tračníku, může tedy ovlivnit jeho motilitu. Avšak nejsou známy žádné studie, které by tento děj detailně popisovaly. Cílem této práce bylo (1) posoudit reaktivitu longitudinálního a cirkulárního svalu distálního tlustého střeva na KCl, ACh a Ang II, (2) charakterizovat receptory zapojené v odpovědi na Ang II a (3) porovnat rozdíly mezi samčím a samičím pohlavím. Experimenty byly prováděny na dospělých potkanech kmene Wistar Han obou pohlaví. Preparáty longitudinální a cirkulární svaloviny z oblasti distálního tlustého střeva byly testovány za pomoci metody standartní orgánové lázně. U obou pohlaví cirkulární sval reaguje na námi testované látky (KCl, ACh, Ang II) více než sval longitudinální. V odpovědi na KCl, ACh a Ang II byly pozorovány rozdíly mezi pohlavími pouze u cirkulárního svalu. Studium vlivu Ang II na hladkou svalovinu distálního tlustého střeva prokazuje, že interakce s AT_1 receptory zprostředkovává její kontrakci, zatímco interakce s AT_2 receptory zprostředkovává její relaxaci.

The results of this work were presented as posters at:

- 9th IJUP (Meeting of Young Investigators of the University of Porto), 17-19 February 2016, Porto, Portugal. Reactivity of rat colon: influence of gender. Coelho, D.C., Kubíčková, R., Morato, M..
- Gordon Research Conference on Angiotensin, 21-26 february 2016, Lucca (Barga), Italy. Rat colonic and mesenteric reactivity to RAS components: influence of gender. Kubíčková, R., Coelho, D., Silva, M., Pires, C., Hutchison, J., Gomes, M., Morato, M..

Content

1 List of abbreviations

ACE	angiotensin converting enzyme
ACh	acetylcholine
ADH	antidiuretic hormone, vasopressin
Ang	angiotensin
AOGT	angiotensinogen
ENS	enteric nervous system
GIT	gastrointestinal tract
IBD	inflammatory bowel disease
KCl	potassium chloride
MrgD	Mas-related G-coupled receptor type D
NA	noradrenaline
NEP	neutralendopeptidase
PEP	propylendopeptidase
RAS	renin angiotensin system

2 Introduction

This work was performed in the Laboratory of Pharmacology, Department of Drug Sciences, Faculty of Pharmacy, University of Porto, under the supervision of Professor Manuela Morato, who carries out long-term research on the distribution and function of several components of the renin-angiotensin system (RAS) in various tissues.

Physiological regulation of the function of the large intestine is neurohumoral. The neural part of this regulation implies the vegetative nervous system, which is crucial for the control of the gastrointestinal tract (GIT) motility. Concerning the humoral part of the regulation, recent studies showed that angiotensin II (Ang II) causes contraction of the colonic smooth muscle and, thus, can also influence the motility of the colon. Since the colon has two layers of smooth muscle, organized in different directions - longitudinal and circular, we decided to compare their reactivity to several drugs between those muscles.

The colon is innervated by sympathetic and parasympathetic nervous system and its motility is the result of their cooperation. Opposite from most other tissues, in the GIT, the parasympathetic nervous system cause contraction of the smooth muscle while the sympathetic nervous system relaxes the smooth muscle. The parasympathetic nervous system controls the motility of the GIT by releasing its mediator – acetylcholine (ACh). Therefore, this work focused on effects of ACh on smooth muscles of the distal colon.

The RAS has been characterized for a long time as a system that regulates the function of blood vessels, heart and kidney, thus with impact on the regulation of blood pressure. Ang II, as the key mediator of the RAS, mediates the classical effects of the system by binding to AT_1 receptors, hence causing vasoconstriction, secretion of aldosterone and retention of water and sodium ions (leading to an increase of blood pressure). Ang II can also stimulate AT_2 receptors that usually mediate opposite effects. This concept of the RAS was further extended to include many other components. Another equally important discovery was the existence of local or tissue RAS, which extended the context from the endocrine to both the paracrine and/or the autocrine level.

In fact, although there are local RAS in several tissues, for example in the nervous system, reproductive organs, the GIT and skin, evidence about its components and functions is sparse. Concerning the GIT, some studies have revealed that in the colon, Ang II regulates sodium and water reabsorption and contracts colonic smooth muscle, thus having an impact on colonic motility. Interestingly, sex hormones seem to influence the production of RAS components as well as the motility of the colon in healthy individuals, although this subject needs further attention.

Another contractile agent, potassium, was studied in the form of potassium chloride (KCl), mainly because the response induced by KCl is receptor independent.

The goal of my master thesis was to compare the reactivity of the longitudinal and circular muscles of the rat distal colon to ACh, Ang II and KCl. Also, we wanted to find out whether that reactivity was different between males and females, and to characterize the receptor mediating the response to Ang II.

3 Theoretical part

3.1 Gastrointestinal tract

3.1.1 Structure and function of gastrointestinal tract

The GIT was phylogenetically developed for the intake of nutrients, liquids, minerals and vitamins. Its functions are digestion (chemical and mechanical processing of food), absorption (transfer of substances into the blood circulation through the GIT wall), and excretion (elimination of undigested residues of food and waste products of metabolism). The GIT begins in the oral cavity and continues along the oesophagus, stomach, small intestine (that consists of the duodenum, jejunum and ileum) and large intestine (caecum, colon and rectum), to terminate at the anus. During its way along the GIT, food is continuously decomposed to the element nutrients that the body can easily absorb. Each part of the GIT has its own specific functions: the oral cavity and the stomach serve mostly for digestion of food, main function of the small intestine is digestion and absorption of nutrients, in large intestine absorption of nutrients still continues but main function is collection and excretion of waste products. These functions of the GIT are influenced by nervous and humoral systems (Rokyta 2000).

3.1.2 Anatomy, histology and physiology of the colon

The large intestine (*intestinum crassum*) consists of 3 main parts (Figure 1):

- The caecum *(intestinum caecum)* is the part that connects with the small intestine; at the caecum there is the vermiform appendix *(appendix vermiformis).*
- The colon is the longest part of the large intestine and it is divided in the ascending colon *(colon ascendens),* attached to the caecum at the right part of the abdominal cavity, the transverse colon *(colon transversum),* which goes under the stomach and liver from the right side to the left side of the body, and the descending colon *(colon descendens),* which goes down at the left side of the body to the small pelvis and continues with sigmoid colon *(colon sigmoideal),* which is S-curved and goes to the middle of the pelvis.

- The terminal part of the large intestine is the rectum that exposes the large intestine to the surface of the body, ending in the anal canal *(anus)* (Čihák a Grim 2002).

These three parts are perfectly enrolled in the abdominal cavity and are linked by the right colic (hepatic) flexure *(flexura coli dextra),* between the ascending and the transverse colon, and the left colic (splenic) flexture *(flexura coli sinistra),* localized between the transverse and the descending colon (Čihák a Grim 2002).

The blood supply to the large intestine is provided by the mesenteric arteries that derive from the aorta, while the venous return from the large intestine is supported by the mesenteric veins that drain to the portal circulation and, then, through the hepatic vein and the inferior vena cava, to the heart.

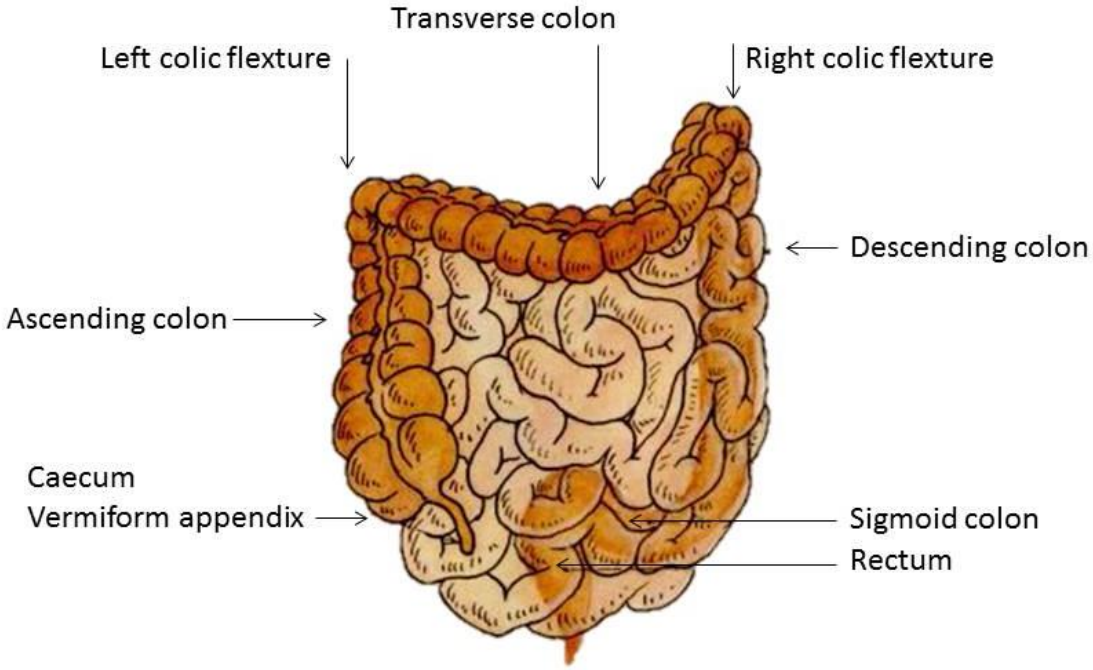

Figure 1 - Anatomy of the large intestine. Adapted from: (Čihák a Grim 2002)

From the distal part of the oesophagus, the GIT wall consists of 5 layers, organized as follow (from the lumen to the peritoneum): mucosa, submucosa, circular and longitudinal smooth muscles, and serosa (Figure 2) (Čihák a Grim 2002).

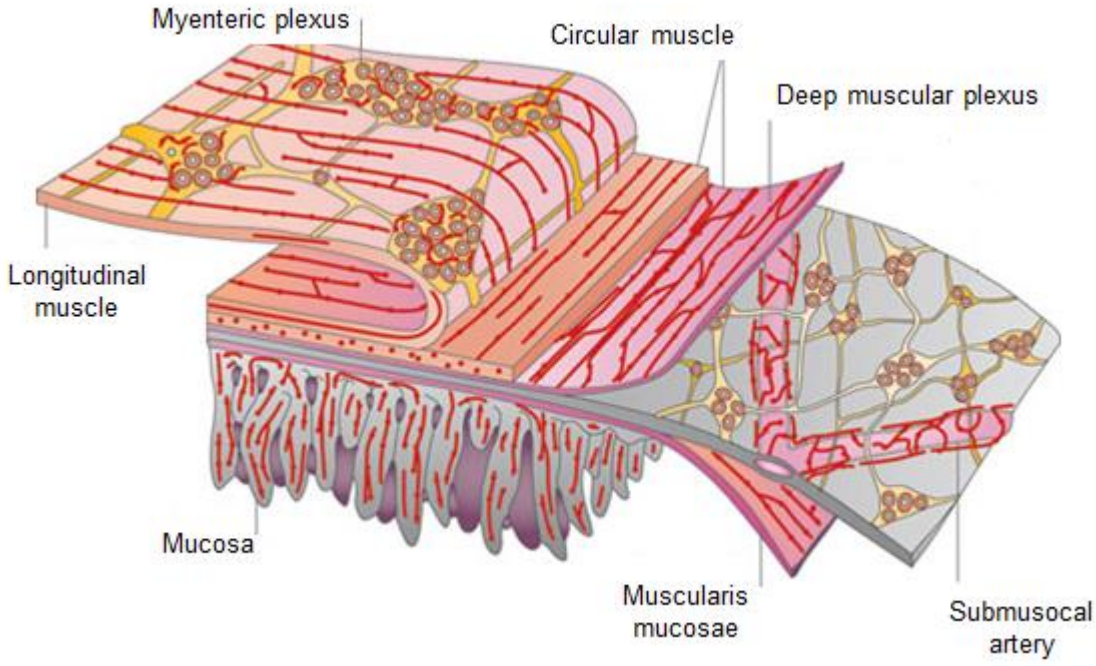

Figure 2 – Histology of the large intestine. Adapted from: (Furness 2012)

In the colon, the mucosa is a thick layer of epithelium formed of columnar absorptive cells and deep tubular crypts (crypts of Lieberkühn) that occupy the whole height of mucosa. Next to the epithelium there is the lamina propria, with the vascular and lymphatic network. Between the crypts of Lieberkühn there is lymphatic tissue formed to lymphatic nodes (Ross et al. 2003). In the mucosa, there are also many goblet cells, which produce the mucus protecting the lumen (Rokyta 2000). The mucosa also includes a muscular layer called *muscularis mucosae*, which is located immediately at the bottom of the crypts of Lieberkühn (Ross et al. 2003).

The submucosa consists of connective tissue and contains vessels, lymphatic nodes and nerves, together in the *plexus submucosus Meissneri* (Ross et al. 2003).

The muscular layer is divided in two: the inner layer, with the smooth muscle cells oriented along the circular axis of the colon, and the outer layer, where the smooth muscle cells are oriented along the longitudinal axis of the colon. Between these two muscle layers is another nerve plexus: the *plexus myentericus Auerbachi.* The longitudinal smooth muscle is thinner than the circular, although it gets thicker in the three *taeniae coli* (Ross et al. 2003).

The most external layer is the serosa, which attaches to the peritoneum and contains vessels.

The main function of the large intestine is to complete absorption and to eliminate undigested residues. In humans, every day, about 1500 ml of chyme arrive to the colon where water, ions as well as bile acids are absorbed. The colon is colonized with symbiotic microflora and this microflora is responsible for the production of vitamins, mainly vitamin K, but also vitamin B_1 and B_2. As a consequence of water absorption, the chyme progressively becomes stiffer, forming the stool that is daily eliminated (about 100–300 g/day) (Rokyta 2000). Stool is eliminated by the function of the smooth muscle layers as well as the internal and external anal sphincters, whose control is involuntary and voluntary, respectively (Rokyta 2000).

3.1.3 Contraction of colonic smooth muscle

The function of the colon depends mostly on the action of the two layers of smooth muscle: longitudinal and circular. Indeed, the longitudinal muscle provides total, propulsive movements (peristalsis) that are important for pushing forward the colonic content. On the other hand, the circular muscle provides local, segmentative and swing movements that contribute to mixing up of the chyme (Rokyta 2000). The colon is innervated by the enteric nervous system (ENS), a complex neural network, considered by some as one of the three subcategories of the autonomic nervous system (Harrington et al. 2010). The tonus of these muscles is controlled by nervous and humoral systems: the *plexus myentericus Auerbachi* controls motility while the *plexus submucosus Meissneri* controls the secretion of mucus and the local blood supply. Both plexus are also connected to sympathetic and parasympathetic nervous system (Rokyta 2000).

So, the colon is innervated by both parasympathetic and sympathetic nervous system, which main neurotransmitters are ACh and noradrenaline (NA), respectively (Rokyta 2000). Differently from most of the other organs, in the colon, ACh binds to ACh receptors (especially muscarinic receptors type 3 (M_3)) on the smooth muscle cells causing contraction (Rokyta 2000). Oppositely, NA activates both α_2 and β_2 adrenoceptors causing relaxation of the smooth muscle cell (Rokyta 2000). Thus, colonic motility is the result of a balance between the sympathetic and parasympathetic effects on smooth muscle cells.

Releasing of ACh from parasympathetic neurons or binding of excitatory mediators (for example endothelin, antidiuretic hormone (ADH), oxytocin, serotonin) cause depolarization of membrane resulting in opening of voltage-gated channels for Ca^{2+} and calcium ions begins to flow into the smooth muscle cell. Inside of the cell, calcium ions bind to the calmodulin and create calcium–calmodulin complexes, which activate myosin light chain kinase. This further causes phosphorylation of myosin fibrous tail, a step that is crucial for contraction. After phosphorylation, globular head of myosin cleaves inorganic phosphor and binds to the binding site of actin polymer (the cross bridge cycle) which is followed by the change of the position of the globular myosin head, which shortens the contractile unit resulting in the contraction of the cell.

3.2 Description of RAS

3.2.1 Classical view of the RAS

The classical view of the RAS is quite simple (Figure 3 and Figure 4). Renin is an enzyme produced and secreted in to the circulation by the juxtaglomerular cells in the kidney (Nguyen a Muller 2010) whose main function is to cleave angiotensinogen, a peptide produced and released by liver, to form Ang I. This decapeptide is then a substrate for angiotensin-converting enzyme (ACE), an enzyme that is mostly present in vascular endothelial cells of the lung. ACE acts as a dipeptidyl carboxylase (Jabor 2008) and converts Ang I to Ang II by cleaving the last 2 amino acids (His-Leu) from the acid tale. Furthermore, ACE can act as a peptidyl dipeptidase and inactivate bradykinin (nonapeptide belonging to the group of kinins with vasodilator and diuretic

effects, which leads to the reduction of the blood pressure) (Bas et al. 2007). Ang II exerts its effects by binding to specific receptors, namely AT_1 and AT_2 receptors.

AT_1 and AT_2 receptors have 7 transmembrane domains and are coupled to G-proteins. Both receptors appear to have the same binding properties for Ang II, but they differ in expression, localization, tissue distribution and mediated actions (Paul et al. 2006). AT_1 receptor mediates most of the well-known actions of Ang II (Figure 5): vasoconstriction and retention of sodium ions and water in proximal tubules and collecting ducts (Paul et al. 2006; Volpe et al. 2003). Oppositely, activation of AT_2 receptors by Ang II seems to trigger opposite responses: vasodilatation and natriuresis (Figure 5) (Volpe et al. 2003). So, the effect of Ang II can be either beneficial or deleterious (Balakumar a Jagadeesh 2014).

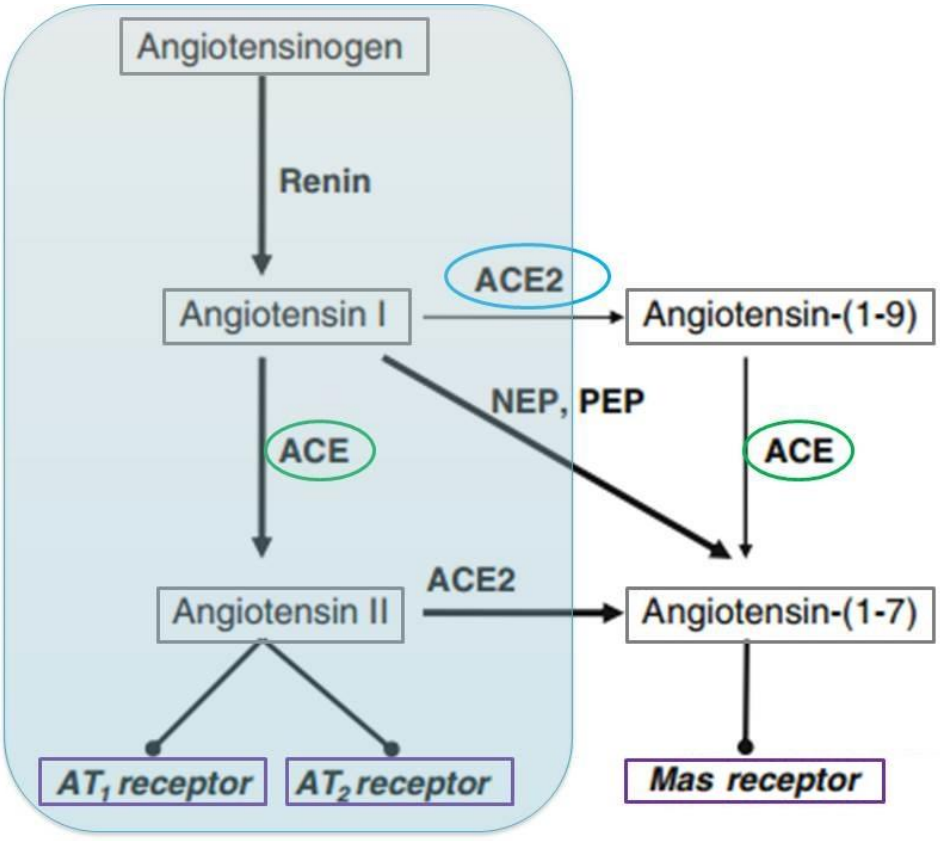

Figure 3 – Schema of RAS. Adapted from: (Bader 2012). In the box is shown classical view of RAS. Outside the box is shown current view of RAS. Abbreviations: ACE – angiotensin-converting enzyme, NEP – neutral endopeptidase, PEP – propylendopeptidase.

Ang II can exert its regulatory functions both directly, through modulation of cardio-renal vascular system, or indirectly, through other regulatory factors, for example by modulation of sympathetic nerve activity (Fändriks 2011; Balakumar a Jagadeesh 2014) or by releasing aldosterone or ADH (Fändriks 2011). The better described effect of Ang II is on vascular tone and thus, on the control of blood pressure. For this also contributes the control of water and sodium balance, by mediating thirst and salt appetite, and by regulating renal sodium and water reabsorption through aldosterone and ADH secretion. The net effect is an increase in the total body fluid and, consequently, in blood pressure.

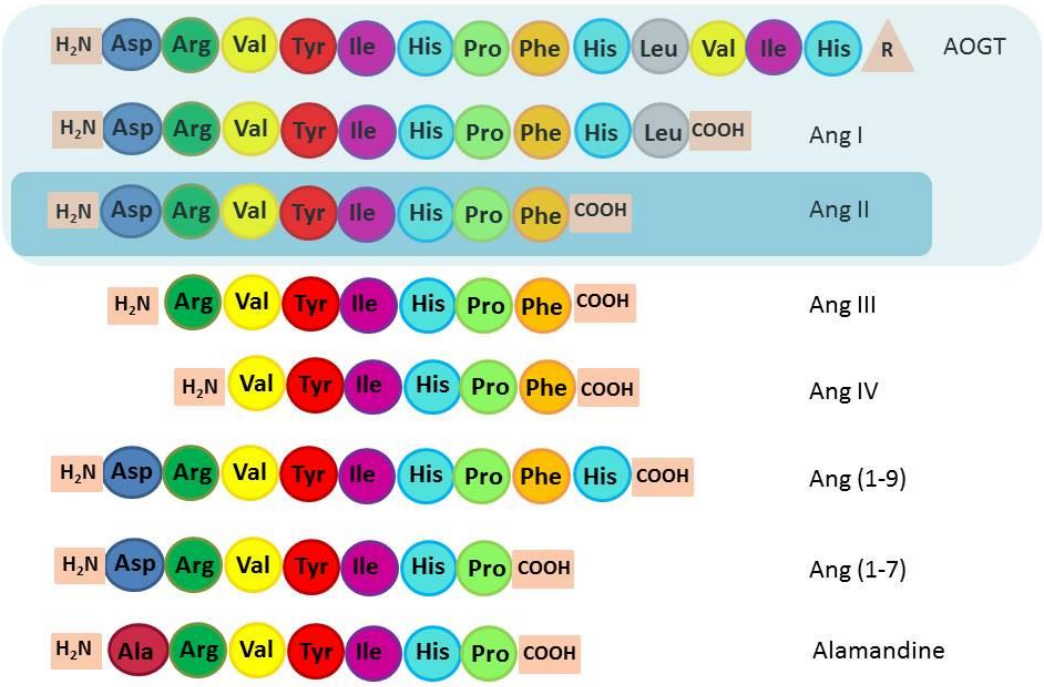

Figure 4 - Structure of angiotensin peptides. In the box is shown the classic view of the RAS with its main mediator Ang II. Outside of the box are newly described angiotensin peptides. Abbreviations: AOGT – angiotensinogen, Ang – angiotensin. Author's own illustration. Based on: (Balakumar a Jagadeesh 2014).

3.2.2 Current view of the RAS

Over the last decades, our knowledge about the RAS has expanded considerably (Figure 3 and Figure 4) as did its role on the cardiovascular system. Indeed, the classic view of the RAS has been enlarged to include several new components and functions, and even a beneficial role for the RAS was revealed.

A precursor of renin was discovered and named as prorenin. Although it was thought to have no function, it is now accepted that its binding to the (pro)renin receptor triggers signalization pathways leading to vasoconstriction, proliferation of smooth muscle cells, collagen production and others, all that independently from Ang II formation (Bultas 2010; Danser 2006). Further, the enzyme ACE2 was described; ACE2 cleaves angiotensinogen to Ang (1-9) and Ang II to Ang (1-7) (Figure 3 and Figure 4) (Paul et al. 2006). It was also revealed that Ang (1-7) is the endogenous ligand of the Mas receptor and that activation of the Mas receptor leads to opposite effects than those of Ang II through AT_1 receptors (Figure 5) (Santos et al. 2013a). Mas receptor is G-protein coupled receptor with 7-transmembrane domains and it is localized in kidney, heart, vessels, brain and testes (Bader 2012). With a similar structure to Ang (1-7), another angiotensin peptide was recently discovered – alamandine. Alamandine binds to the Mas-related G-coupled receptor type D (MrgD) receptor and have similar actions to those of Ang (1-7) (Balakumar a Jagadeesh 2014). Its expression is suggested in muscle, heart and testicle (Villela et al. 2014).

Ang II is the key mediator of the RAS and it is also the most described peptide of the whole RAS axis. Although the most explored pathway to form Ang II is the cleavage of Ang I by the action of ACE in the lungs, there is also an alternative pathway, through which Ang II can be formed by the action of chymase, an enzyme secreted by activated mast cells in heart and vascular wall (Takai et al. 1999). Recently, other angiotensin peptides – Ang III and Ang IV were identified. Ang III, also known as angiotensin (2-8), is formed from Ang II by action of aminopeptidase A and has similar actions to Ang II on AT_1 and AT_2 receptors (Garg et al. 2012). Ang IV, also known as angiotensin (3-8), is formed from Ang III by the action of aminopeptidase B or N and shows reverse effects to those of Ang II (Garg et al. 2012).

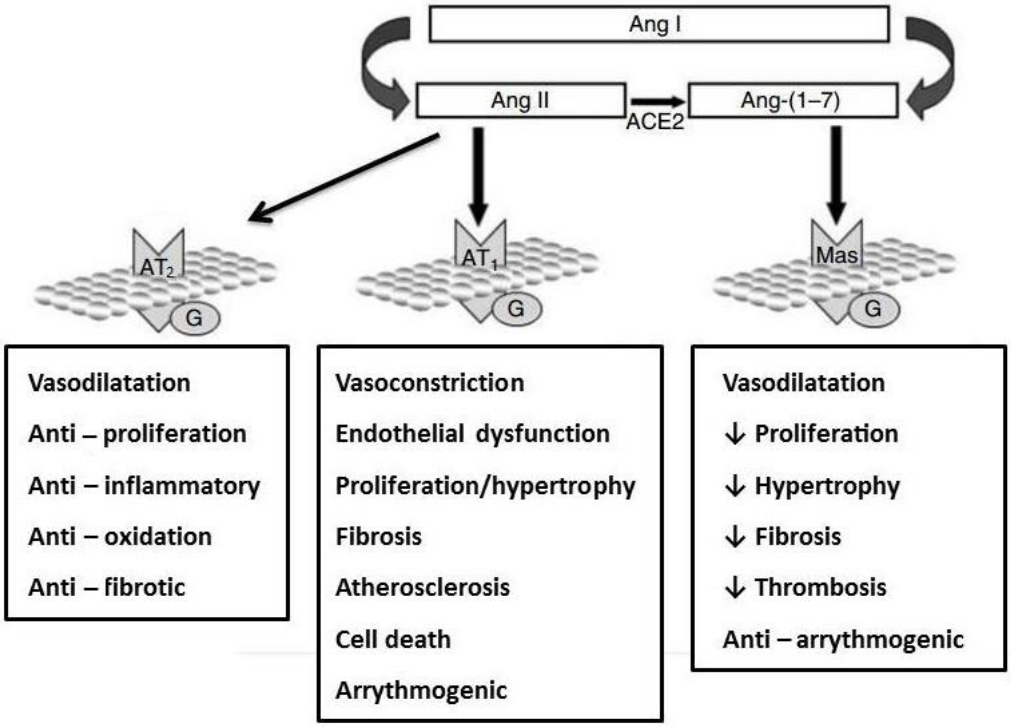

Figure 5 – Actions mediated by AT_1, AT_2 and Mas receptors. Adapted from: (Santos et al. 2013b).

3.2.3 Angiotensin II

The function of Ang II was recently expanded outside of the cardiovascular and renal systems. Ang II can affect the function of many organs, which are not connected with blood pressure control. So, Ang II can also regulate functions in the brain, GIT or reproductive organs (Balakumar a Jagadeesh 2014). In the brain Ang II is associated with alteration of the cerebral blood flow and pro-inflammatory states (Kalra et al. 2015), while in reproductive organs Ang II is associated with alterations in uterine blood flow in females and percentage, motile and velocity of sperm in males (Paul et al. 2006). Besides the endocrine functions of Ang II, it has also functions at the cellular level, as cell proliferation, differentiation, growth, and apoptosis (Paul et al. 2006). Moreover, Ang II can play a role in inflammatory processes, carcinogenesis and generation of reactive oxygen species (Mastropaolo et al. 2013). Regarding the GIT, it was reported that Ang II can modulate contractile activity in the murine colon, more specifically it can enhance spontaneous contractile activity and cause releasing of

tachykinins and ACh (Mastropaolo et al. 2013). Also role of Ang II in pathophysiology of several diseases of the GIT has been shown: Ang II can contribute to the development of inflammatory bowel diseases (IBD) and irritable bowel syndrome, as well as functional diseases as incontinence of the internal anal sphincter (Hadzhibozheva et al. 2013).

3.3 RAS in the colon

The presence of RAS components in different tissues or organs is now well accepted (Paul et al. 2006). Some tissues are able to synthetize their own components of the RAS, highlighting its relevance as an endocrine, paracrine and autocrine system, or, alternatively, RAS components can be taken up from the circulation (Paul et al. 2006). Local RAS have been described in many tissues, as the kidney, liver, pancreas, nervous system, reproductive organs, skin and digestive organs (Fyhrquist a Saijonmaa 2008; Paul et al. 2006). This concept suggest the importance of local RAS in maintaining tissue homeostasis through regulation of blood flow, electrolyte and fluid balance, proliferation, fibrosis, protein synthesis and inflammation (Garg et al. 2012).

Ang II can play a role in the absorption or secretion of water and electrolytes to maintain total body fluid and electrolyte balance as well as it can influence colon motility through its effect on smooth muscle. So, it is greatly important to study the effects of RAS in the GIT. ACE is present in the intestine brush-border membrane and can contribute to the digestion of dietary peptides (Paul et al. 2006). Moreover, dysfunction of the RAS plays a role in the development of IBD, for example Crohn's disease (Garg et al. 2012).

Transcripts encoding renin, angiotensinogen, ACE, ACE2 and AT_{1A} have been detected in the entire length of the colon, while AT_2 is reported to be expressed only in the distal part of colon (Mastropaolo et al. 2013; Garg et al. 2012).

However, we do not know much about the functions of the RAS in the colon. It is known that Ang II enhances sodium and water reabsorption through NaCl-coupled transport and that Ang II contracts circular and longitudinal muscle of the human colon, thus being able to influence colon motility (Garg et al. 2012). This effect of Ang II on

colonic contractility occurs in concentrations similar to those found in the plasma (Mastropaolo et al. 2013). Thus, Ang II receptors can be targeted either by circulating Ang II or by locally produced Ang II, highlighting its endocrine and paracrine role on colon contractility (Mastropaolo et al. 2013).

3.4 Gender differences

Males and females of each species differ in many conditions. The basic item is composition and structure of the body. Gender determines the amount of muscle tissue, quantity and distribution of fat tissue as well as the volume of body fluid. In general, we know that males have more robust bodies, with more muscle and less fat, have greater weight and height. The differences are highly dependent on the reproductive organs, secondary sexual characteristic and sex hormones, which influence physical as well as psychological aspect of the individuals: while in males the levels of hormones are stable along their fertile period, in females the levels of sexual hormones fluctuate throughout a cycle. Because of these gender differences, some physiological conditions can be different and each gender can be more prone to specific pathophysiological conditions and diseases.

Concerning the GIT, females generally have slower colonic transit time than males (Meier et al. 1995). Furthermore, gender can influence the bacterial community of the colon, which might contribute to different immunity and resistance to disease (Lumpkins et al. 2008). Regarding diseases of the GIT, gender influences the prevalence of irritable bowel disease, since approximately two thirds of the patients are females. This difference might be associated with some physiologic characteristics as visceral sensitivity, GIT transit time, central nervous system pain processing or effects of sex hormones on gut function. Interestingly, females report different symptoms of IBD than males: females typical complain about nausea, bloating and constipation while males most usually report diarrhoea (Chang a Heitkemper 2002). Also drug responsiveness is influenced by gender (Chang a Heitkemper 2002). Moreover, the bodily pain scores are higher in females than in males. This finding is not unique, since overall, females have higher predominance for chronic painful disorders and, in many cases, IBD is connected with another female-predominant disease such as chronic

constipation, chronic pelvic pain, chronic fatigue syndrome, migraine headache with aura, depression and anxiety (Chang a Heitkemper 2002). These last three diseases are influenced by the levels of serotonin which is lower in females compared to males (Nishizawa et al. 1997). As we can see, females are affected by some diseases more than males.

Currently, only some studies describe how RAS components differ between males and females. It can be said that oestrogens increase the levels of angiotensinogen in plasma and the mRNA levels of angiotensinogen in the liver and kidney, while decrease plasma levels of renin and ACE activities, although the effect varies with the way of administration of oestrogens (Komukai et al. 2010). Although no influence of oestrogens on Ang II levels has been described yet, they decrease the density of AT_1 receptors (Komukai et al. 2010). Thus it seems, that oestrogens play protective role against overactivation of RAS and connected cardiovascular disorders.

Unfortunately, in the GIT, namely in the colon, gender differences concerning RAS components are not so well characterized yet. On the other hand, as previously said, females are more prone to some gastrointestinal inflammatory diseases and the colonic transit time differs between males and females. However, since RAS seems to play a role in GIT motility and gender has impact on RAS components, new studies are needed in order to clarify how can RAS influence GIT function and also how can gender influence that effect of RAS.

4 Aims

The aims of this work were:

- To compare the reactivity of the longitudinal and circular smooth muscles of the rat distal colon to KCl, ACh and Ang II

- To compare the reactivity of the colonic smooth muscles to those drugs between males and females

- To characterize the receptors involved in the response of the colon to Ang II

5 Materials and methods

5.1 Chemicals and preparation of solutions

Most of the compounds used for the experimental work were obtained from Sigma-Aldrich, USA except for: NaCl (VWR chemicals, Belgium), KCl, $CaCl_2$, glucose, $NaHCO_3$, ascorbic acid (Pancreac Quimica, Spain), $NaH_2PO_4 \times H_2O$, Na_2EDTA (MERCK, Germany). Candesartan was a kind gift from Dr. Fredrik Palm (Uppsala University, Sweden).

- ACh – powder ACh chloride was dissolved in distilled water to the needed concentrations. Stock solution was prepared fresh each week and stored in the fridge.

- Ang II was dissolved in distilled water to 10^{-2} M and stored in 10 µl aliquots, at -20°C. For each experiment, this stock solution was diluted with distilled water to have concentrations of Ang II of 10^{-3} M, 10^{-4} M, 10^{-5} M, 10^{-6} M and 10^{-7} M. Finally, to obtain the wanted concentration in the organ bath, a certain volume of the dilution was added to the organ bath (Appendix 1).

- Candesartan powder (8.8 mg) was dissolved in a mixture of physiological saline (0,9% NaCl; 450 µl) plus Na_2CO_3 (0.5M; 50 µl) and stirred until totally dissolved. Then, the volume was completed adding physiological saline (0,9% NaCl; 1.5 ml). The solution was stored in 10 µl aliquots, at -20°C. Finally, to obtain the wanted concentration in the organ bath, a certain volume of the dilution was added to the organ bath (Appendix 1).

- PD 123319 was dissolved in distilled water to 10^{-2} M and stored in 10 µl aliquots, at -20°C. To obtain the wanted concentration in the organ bath, a certain volume of the dilution was added to the organ bath (Appendix 1).

- Krebs solution – Krebs solution was prepared according Table 1 using distilled water as solvent.

Table 1 - Preparation of Krebs solution

Compound	g/l	mM
NaCl	69	118
KCl	3,6	4,8
$CaCl_2$	2,8	2,5
$MgSO_4$	2,96	1,2
NaH_2PO_4	1,65	1,2
Glucose	2,2	11
$NaHCO_3$	2,1	25
Na_2EDTA	0,0112	0,03
Citric acid	0,0528	0,3

- Krebs solution + KCl – was prepared according Table 2 using distilled water as solvent.

Table 2 - Preparation of Krebs solution with KCl 125 mM

Compound	g/l	mM
KCl	93,2	125
$CaCl_2 \times 2H_2O$	2,8	2,5
$MgSO_4 \times 7\ H_2O$	2,96	1,2
$NaH_2PO_4 \times 2H_2O$	1,65	1,2
Glucose	2,2	11,1
$NaHCO_3$	2,1	25
Na_2EDTA	0,0112	0,03

5.2 Animals and tissue preparation

Experiments were performed using male and female Wistar Han rats (ICBAS-Porto animal facility, Portugal) at the age of 10-12 weeks. The animals were housed in standard conditions of temperature (21ºC), humidity (40-60%) and alternative cycles of light and dark (12h/12h), with free access to food and water. All experiments were performed in accordance with the European Union guidelines for the protection of animals used for scientific purposes (Directive 2010/63/EU and Decision 1999/575/EC) and national guidelines (DGAV, Portugal). Animals were sacrificed by decapitation (using guillotine) and after a midline laparotomy a part of the distal colon (approximately 4 cm) was excised and gently flushed with Krebs solution to get free of

luminal content. Each colon was opened and cut in segments of about 20 mm long, which were oriented along the longitudinal and transversal muscle fibres, and mounted in 20 ml organ baths filled with warm (37°C) oxygenated (95% O_2, 5% CO_2) Krebs solution (Appendix 3). A cotton thread was used to fix the tissues to the bottom of the bath, while another fixed the tissue to a transducer (UGO BASILE S.R.I., Italy, Model 7004). Isometric responses were recorded on a polygraph (UGO BASILE S.R.I., Italy, Model 7050 and 7070). The tissues were stretched passively to an initial tension of 1g.

5.3 Experimental protocol

At the beginning of each experiment, tissue fragments were allowed to equilibrate to develop spontaneous contractions for at least 30 minutes. In addition, tissues were exposed to ACh (10μM), then ACh was washed away and tissue was equilibrated for 30 minutes (washing every 10 minutes). After this time, the same concentration of ACh was tested (or, eventually, a third time) until the response was stable. After that, a cumulative curve to ACh was performed with following concentrations: 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1 μM, 3 μM, 10 μM, 30 μM, 100 μM, 300 μM, 1 mM and 1,7 mM. These concentrations were chosen to obtain full concentration curve with minimum and maximum response. After 30 minutes of washing, a non-cumulative concentration-response curve was obtained for Ang II. For that, the following concentrations were tested: 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1 μM and 3 μM. Between two concentrations of Ang II the tissues were washed for 1 hour (washing every 15 minutes) to avoid taquiphylaxis. At the end of the experiment, a response to KCl 125 mM was obtained and considered as the maximum response of the tissue. Finally, the tissues were removed from the organ baths, dried on filter paper and weight. The experiment was finished with the sensitivity calibration to every magnification that has been used along the protocol.

When the AT_1 and AT_2 receptor antagonists were tested, at the beginning of each experiment, the tissue was allowed to equilibrate and the concentration of 10 μM ACh was tested, until the response was stable. Then in the longitudinal and in the circular muscle, the response to 30 nM and 300 nM Ang II was tested, respectively. Ang II was

washed away and tissue was equilibrated for 1 hour (washing every 15 minutes). Then the tissue was pre-incubated with candesartan (1 nM or 10 nM) and/or with PD123,319 (1 μM or 10 μM) without washing. After 30 minutes the same concentration of Ang II was tested (30 nM longitudinal muscle, 300nM circular muscle). At the end of the experiment the procedure was the same as the previous protocol, it means measuring of the response to KCl 125 mM considered as the maximum response of the tissue, then the dried tissues were weight and the calibration of the sensitivity of the setup was performed.

5.4 Expression of results and statistical analysis

The height of each response was measured (example of the records in Appendix 2), with a ruler, in millimetres and converted to grams according to the calibration performed at the end of the experimental protocol (for an example, see Appendix 2). Since the response (force) of the tissue is dependent of the amount of smooth muscle and this is indirectly related to tissue weight, the force of contraction was normalized to tissue weight and, thus, results are expressed as g of tension per g of tissue (for an example, see Appendix 2). Results were also expressed as % of the response to KCl 125 mM, which, as previously said, was considered to represent the maximum response of the tissue.

The individual responses of each tissue to the different drugs tested were used to construct concentration-response curves using the GraphPad Prism 6.0 software and to obtain the correspondent E_{max} (g/g), E_{max} (%KCl) and EC_{50} (nM) values. Student's t test unpaired was used to compare means between two groups (either longitudinal vs circular muscle or male vs female). Student's t test paired was used to compare means between two groups (preparations without pretreatment vs pretreated with antagonists of AT_1 and/or AT_2 receptors), and a p value lower than 0.05 was considered to note statistically significant differences.

6 Results

The body weight of male rats was higher than that of female rats (341 ±5 g vs 188 ±3 g, respectively, $p<0.05$). The weight of the strips of longitudinal muscle was significantly higher that of those of circular muscle both in males (51±3 mg vs 28±2 mg, respectively, $p<0.05$) and in females (39±4 mg vs 22±1 mg, respectively, $p<0.05$). Also, the strips of male rats were heavier than those of female rats for both the longitudinal (51±3 mg vs 39±4 mg respectively, $p<0.05$) and the circular (28±2 mg vs 22±1 mg, respectively, $p<0.05$) muscle.

6.1 Reactivity to KCl, ACh and Ang II

6.1.1 Circular versus longitudinal muscle in male rats

In males, the contraction of the circular muscle was higher than that of the longitudinal muscle for every tested drug (KCl, ACh and Ang II) (Figure 6A and Table 3). For ACh, this difference was observed when the response was expressed either in g/g or in % of KCl (Figure 6B and Table 3). However, the response to Ang II was also higher for the circular muscle than for the longitudinal muscle. This difference was only observed when contraction was expressed as g/g (Figure 6A), and not as % of KCl (Figure 6B and Table 3). In males, the EC_{50} values for ACh (Figure 7A and Table 3) and for Ang II (Figure 7B and Table 3) were higher for the circular muscle than for the longitudinal muscle.

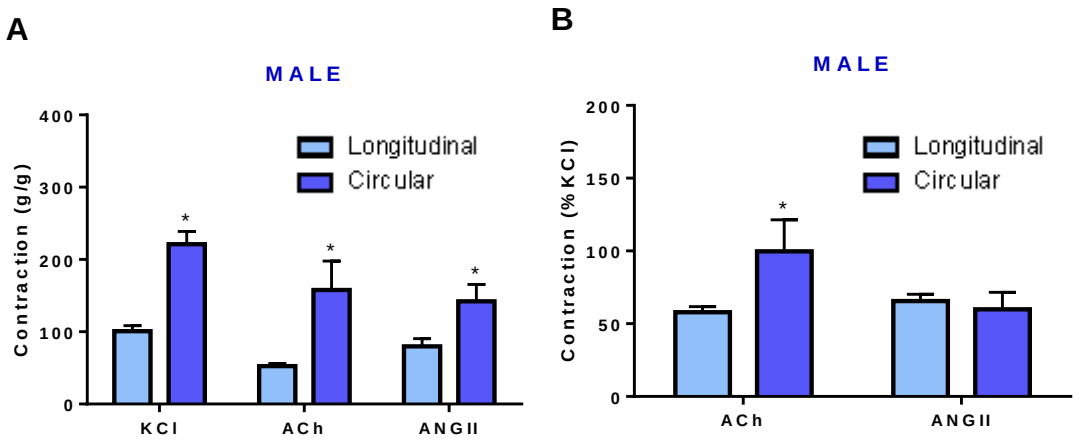

Figure 6 - Reactivity of the longitudinal and circular smooth muscle of the male rat distal colon to KCl, ACh and Ang II. A) Results in g of force/g of tissue; B) Results in % of KCl. Asterisk marks samples with significant difference ($p<0.05$) (evaluated by Student's t test unpaired).

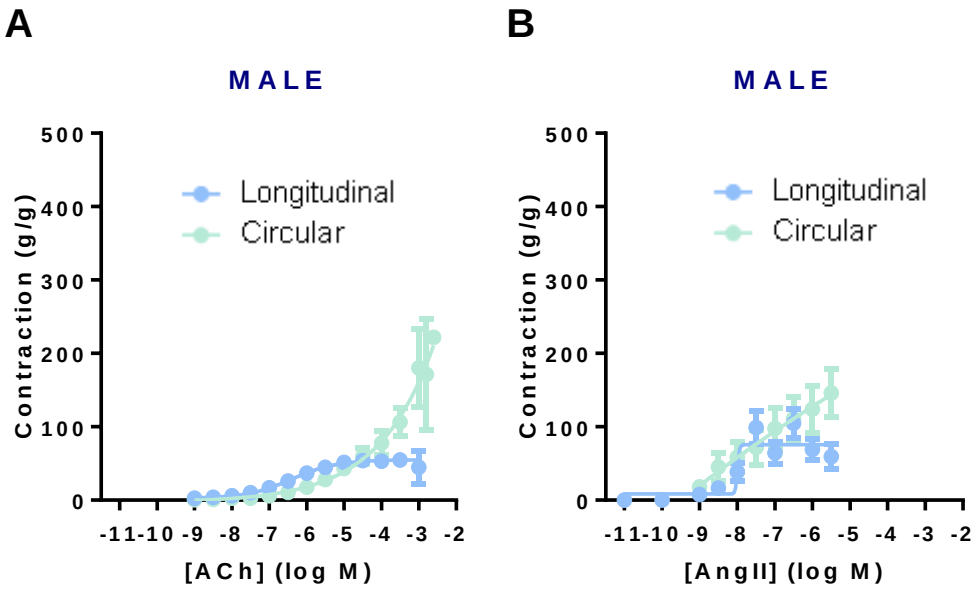

Figure 7 – Concentration-response curves for ACh (A) and Ang II (B) in the longitudinal and circular smooth muscle of the male rat distal colon.

6.1.2 Circular versus longitudinal muscle in female rats

In females, similarly as observed in males, the contraction of the circular muscle was higher compared to longitudinal muscle for every tested drug (Figure 8A and Table 3). For ACh, the response was higher in the circular muscle compared to longitudinal muscle but only when contraction was expressed as g/g (Figure 8A and Table 3), and not as % of KCl was used (Figure 8B and Table 3). The response to Ang II was significantly higher in the circular muscle compared to the longitudinal muscle when expressed as g/g (Figure 8A and Table 3), but when the results were expressed as % of KCl, the response of the circular muscle was lower compared to the longitudinal muscle (Figure 8B and Table 3).

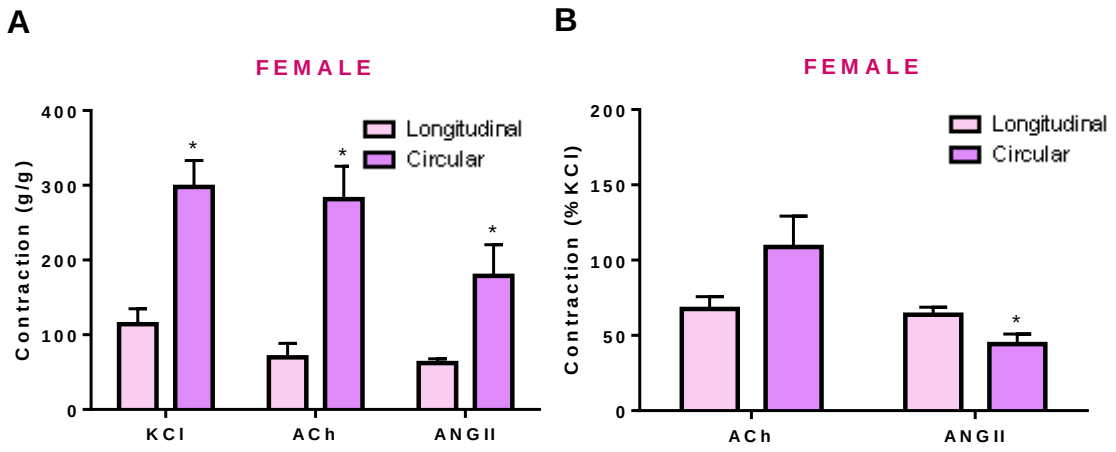

Figure 8 - Reactivity of the longitudinal and circular smooth muscle of the female rat distal colon to KCl, ACh and Ang II. A) Results in g of force/g of tissue; B) Results in % of KCl. Asterisk marks samples with significant difference ($p<0.05$) (evaluated by Student's t-test unpaired).

The EC_{50} for ACh on the circular muscle of female rats was higher compared to the longitudinal muscle (Figure 9A and Table 3). The EC_{50} for Ang II on the circular muscle was also higher compared to the longitudinal muscle (Figure 9B and Table 3).

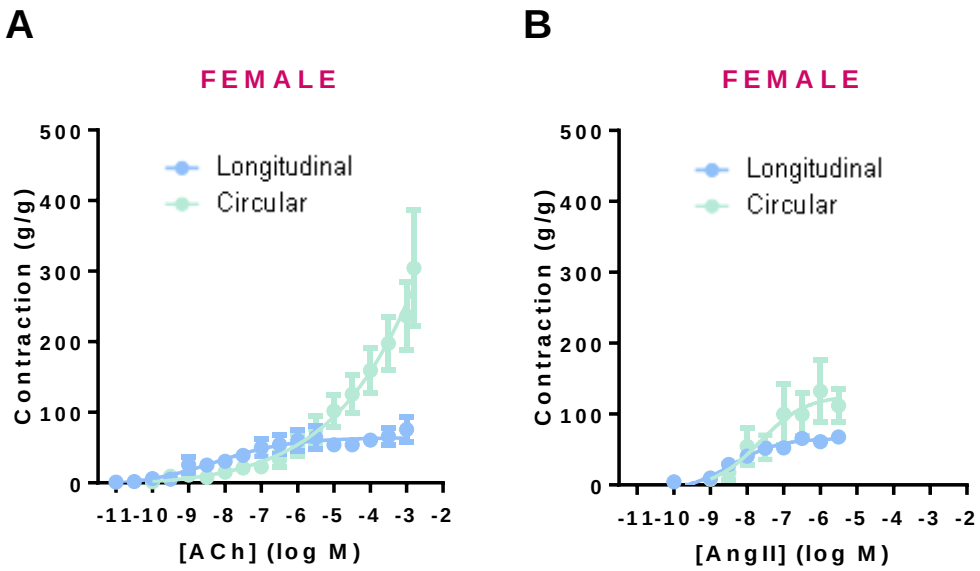

Figure 9 – Concentration-response curves for ACh (A) and Ang II (B) in the longitudinal and circular smooth muscle of the female rat distal colon.

6.1.3 Males / females comparison

In the longitudinal muscle, there was no difference in the response to KCl between males and females (Figure 10 and Table 3).

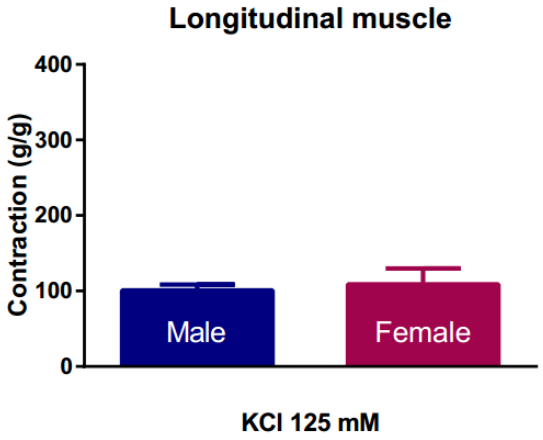

Figure 10 - Reactivity (g/g) of the longitudinal smooth muscle of the male and female rat distal colon to KCl.

For ACh (Figure 11A and Table 3) and Ang II (Figure 11B and Table 3) there was no difference in the response of the longitudinal muscle between males and females.

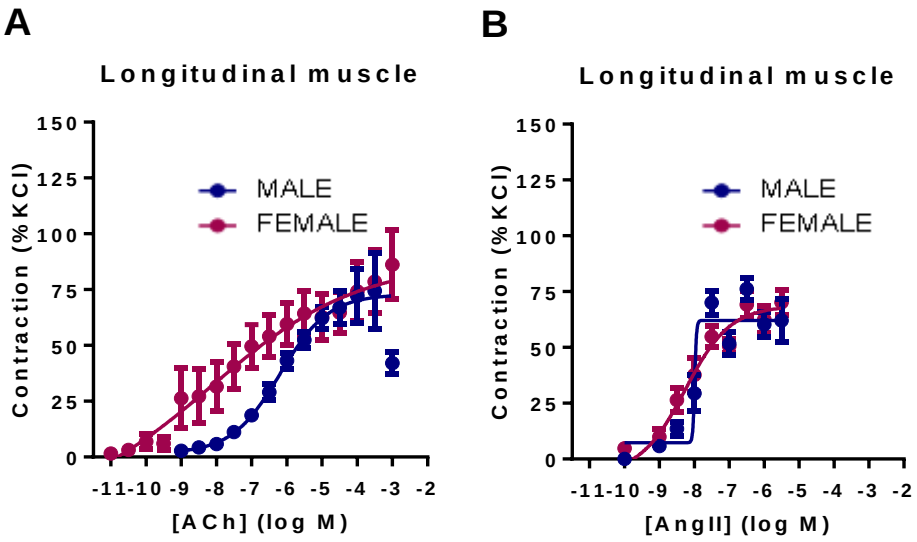

Figure 11 – Concentration-response curves to ACh (A) and Ang II (B) in the longitudinal smooth muscle of male and female rat distal colon.

In the circular muscle, the response to KCl was significantly higher in female compared to male rats (Figure 12 and Table 3). Moreover, the response to ACh was also higher in females but only when the response was expressed as g/g and not as %KCl (Figure 13A and Table 3). There was no difference in the response of the circular muscle to Ang II between genders (Figure 13B and Table 3).

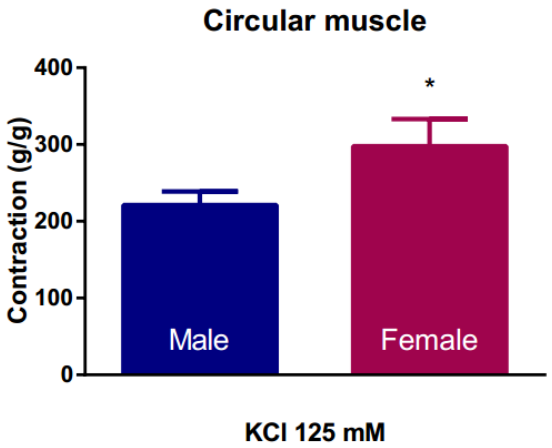

Figure 12 - Reactivity (g/g) of the circular smooth muscle of the male and female rat distal colon to KCl. Contraction of female circular muscle was significantly higher (* $p<0.05$) (evaluated by Student's t test unpaired).

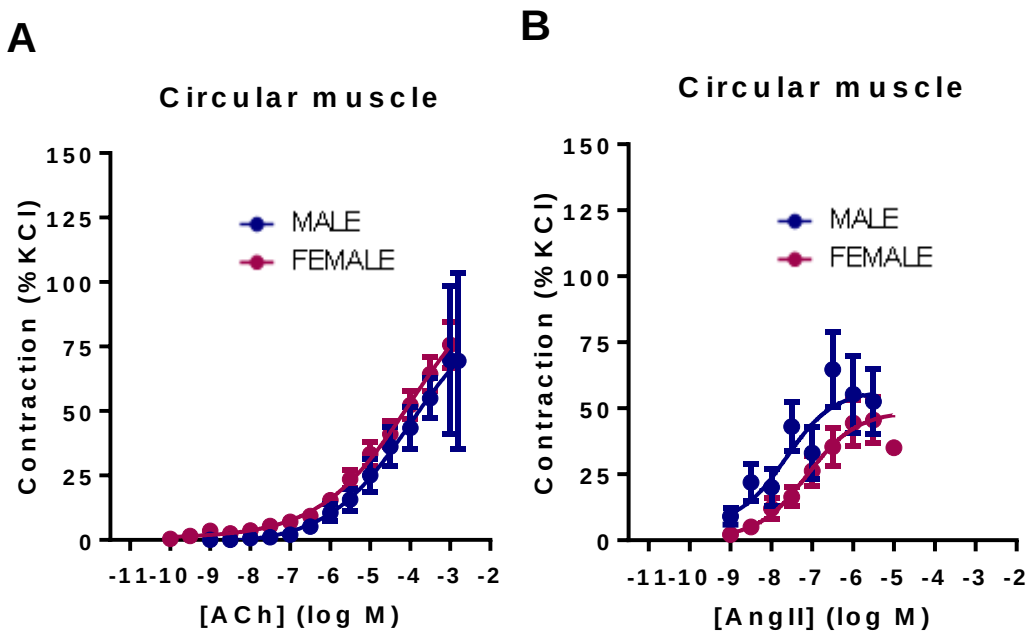

Figure 13 – Concentration-response curves to ACh (A) and Ang II (B) in the circular smooth muscle of male and female rat distal colon.

Table 3 - Reactivity of the male and female longitudinal and circular smooth muscle of the rat distal colon to KCl, ACh and Ang II

				longitudinal muscle	circular muscle
Males ♂	KCl	E_{max}	(g/g)	100.88±7.52	221.04±17.91*
	Ach	E_{max}	(g/g)	52.44±3.65	157.97±39.95*
			% KCl	57.99±3.74	99.69±21.76*
		EC_{50}	µM	2.12±0.98	59.06±27.53*
	Ang II	E_{max}	(g/g)	79.71±10.90	142.09±23.48*
			(g/g)	65.61±4.72	59.93±11.65
		EC_{50}	nM	9.83±3.07	47.79±17.25*
Females ♀	KCl	E_{max}	(g/g)	108.97±15.06	297.90±35.31*,#
	Ach	E_{max}	(g/g)	51.78±4.48	296.15±44.55*,#
			% KCl	67.63±8.04	108.69±20.51
		EC_{50}	µM	0.04±0.01	106.87±35.27*
	Ang II	E_{max}	(g/g)	62.08±5.91	178.94±41.57*
			(g/g)	63.82±4.98	44.25±6.63*
		EC_{50}	nM	5.44±1.08	71.42±20.49*

Results are represented as mean±s.e.m.; * $p<0.05$ vs data obtained in the correspondent longitudinal muscle; # $p<0.05$ vs data obtained in the correspondent male data (evaluated by Student's t test unpaired).

6.2 Characterization of the receptor for Ang II

6.2.1 Ang II in male rats

In male rats, the response to Ang II was significantly attenuated in both, the longitudinal (Figure 14A) and circular (Figure 14B) smooth muscle preparations, in the presence of 1 nM of the candesartan (AT_1 receptor antagonist). When AT_1 receptors were blocked using 10 nM candesartan, the response of the longitudinal smooth muscle to Ang II was totally abolished (Figure 14A) and the response of the circular smooth muscle was almost abolished (Figure 14B).

Pre-incubation of both the longitudinal (Figure 14A) and circular (Figure 14B) smooth muscle preparations with 1 μM PD123,319 (selective AT_2 receptor antagonist) led to increase of response to Ang II compared to native state. Increasing the concentration of PD123,319 (10 μM) also increased the response to Ang II in the longitudinal (Figure 14A) but not in the circular (Figure 14B) muscle of male rats. Blockade of both AT_1 and AT_2 receptors abolished the response to Ang II in the longitudinal (Figure 14A) colonic smooth muscle and almost abolished it in the circular (Figure 14B) colonic smooth muscle of male rats.

6.2.2 Ang II in female rats

In female rats, the response to Ang II was also attenuated in the longitudinal (Figure 14C) and circular (Figure 14D) muscle preparations blocking AT_1 receptors (1 nM candesartan). When the AT_1 receptors were blocked with 10 nM candesartan, the response of both the longitudinal (Figure 14C) and circular (Figure 14D) smooth muscle to Ang II was totally abolished.

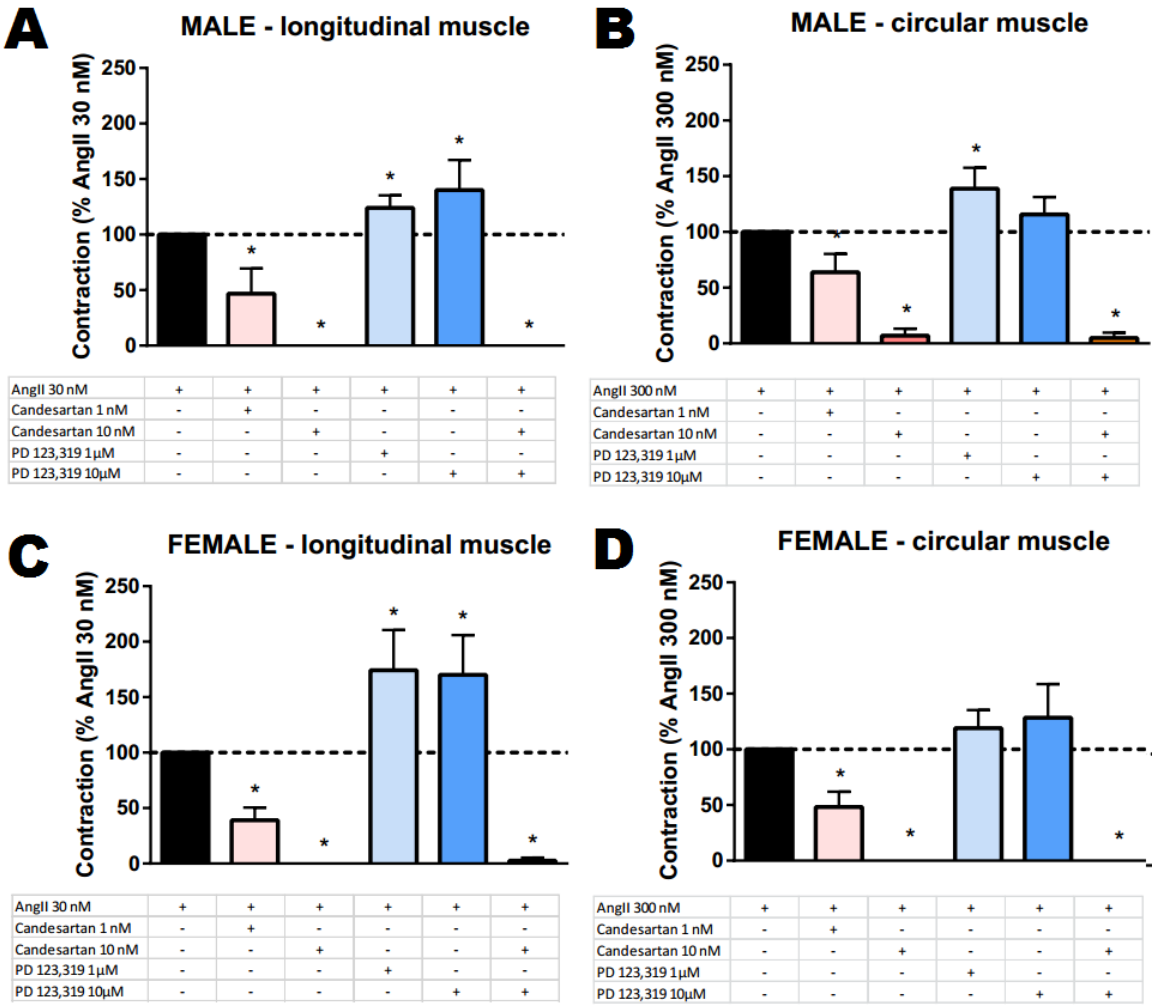

Figure 14 - Response of the longitudinal (A, C) and circular (B, D) smooth muscle of the male (A, B) and female (C, D) rat distal colon to Ang II in the absence and in the presence of candesartan (1 and 10 nM), PD 123,319 (1 and 10 µM) or both (10 nM candesartan plus 10 µM PD 123,319). Results expressed as % of response in the absence of the antagonists. Asterisk marks samples with significant difference ($p<0.05$) (evaluated by Student's t test paired).

Blocking the AT_2 receptors (PD123,319, either 1 µM or 10 µM) increased the response to Ang II in the longitudinal (Figure 14C) colonic smooth muscle of female rats, when compared with the response of Ang II alone. However, the PD 123,319 had no effect on the response to Ang II in the circular (Figure 14D) colonic smooth muscle of female rats.

Blockade of both AT_1 and AT_2 receptors almost abolished the response to Ang II in the longitudinal (Figure 14C) colonic smooth muscle and abolished it in the circular (Figure 14D) colonic smooth muscle.

7 Discussion

The results of the present study highlight differences between the longitudinal and the circular smooth muscle of the rat distal colon and also between males and females. Moreover, these results point to different relevance of Ang II receptors in mediating the response to Ang II in the rat distal colon.

The results show that the circular smooth muscle of the rat distal colon has higher capacity of the contraction and that the response to the tested substances of the circular muscle was higher compared to the longitudinal muscle. One of the possible reasons explaining this finding could be connected to different number of muscle layers between circular and longitudinal muscle as more layers and bigger size of the smooth muscle can be found in the circular muscle tissue. To avoid false positive or negative results, when comparing directly circular and longitudinal muscle in our experiments, this morphological difference was overcome by normalization to the response of the 1 g of the muscle tissue. Concerning the initial tension, during preliminary experiments, various initial tensions were tested and then the best one (1 g) for both longitudinal and circular muscle was chosen. Thanks to the influence of the inappropriate initial tension was also eliminated. Additionally, the calcium dependence of the smooth muscle is worth consideration as it is known that contraction of cells isolated from the circular smooth muscle of the human colon is highly dependent on extracellular calcium (Boyer et al. 2001). If we could extrapolate the results of that study to our experimental setup, the circular smooth muscle would mobilize more extracellular calcium ions than the longitudinal smooth muscle and then, when stimulated, it would develop more extensive contraction. Further possible explanation of the differences between the longitudinal and the circular muscle could be the different modification of the signal transduction. In the circular muscle, modification of the signal transduction could be different with an involvement of other mediators and receptors. Thus, the clarification about the signal transduction in the longitudinal and the circular smooth muscle of the rat distal colon is needed. Another possible explanation of our results could be the different density or quantity of the receptors for ACh and Ang II, or involvement of different subtypes of the receptors for ACh and Ang II. Further experiments are also needed to clarify these issues, namely by evaluating receptor expression by immunohistochemistry or

characterizing the signal transduction mechanisms testing several inhibitors, in organ baths functional assays.

Ovarian hormones can influence sensorymotor activity of the GIT in the healthy population (Yang et al. 2014). Also, sex hormones can influence the colonic transit time. Indeed, Chang a Heitkemper (2002) reported that women show overall longer colonic transit time compared to men. Meleine a Matricon (2014) characterized two different patterns of colonic transit in women dependent on oestrogen levels. During the stages of the hormonal cycle with the elevated levels of oestrogens the colonic transit is slowed down compared to stages with the elevated progesterone levels. Colonic transit time is mostly associated with contraction of the longitudinal smooth muscle, which main function is the stimulation of propulsive movements in order to push forward the colonic content. However, we found no differences between genders concerning the reactivity of the longitudinal muscle of the distal colon to any of the drugs tested. These findings suggest that the gender-associated differences in colonic transit are not a direct consequence of alterations in longitudinal smooth muscle reactivity. Eventually, other parts of the colon, like the proximal part, could reveal different results than those reported by us in the distal colon. For our experiments, we did not consider the stage of the estrous cycle in female rats thus we don't know the precise levels of sex hormones by the time of the experiment. If the female rats used in this study were in very different phases of the estrous cycle, the levels of sex hormones could vary considerably and this fact could influence our results. However, our results are stable and so, not knowing the stage of the estrous cycle of the female rats, we don't think that they were influenced by the varying hormone levels. However, in the future studies, this technical handicap will be solved and the stage of the estrous cycle will be evaluated in the female rats to confirm or disprove our findings.

Sex hormones also influence the expression of RAS components as supported by the finding of oestrogen-responsive element in the angiotensin gene (Paul et al. 2006). Oestrogens increase the plasma levels and liver and kidney mRNA levels of AOGT (Komukai et al. 2010) while decreasing the levels of other RAS components, namely plasma levels of renin, serum ACE activity and their mRNA expression (Paul et al. 2006). Although the sex hormones do not alter Ang II levels, AT_1 receptors are downregulated by oestrogens. Testosterone induces the opposite effect (Paul et al. 2006; Komukai et al. 2010). If this decreased expression of AT_1 receptors induced by

oestrogens was regionally targeted at the circular smooth muscle, it could justify the decreased reactivity of the female colonic smooth muscle to Ang II. However, our results about the characterization of the receptors involved in the response to Ang II don't fit with this hypothesis.

The results with the antagonists of Ang II receptors show that in both, the longitudinal and the circular smooth muscles of the rat distal colon, Ang II-mediated contraction is mediated through AT_1 receptors. This response to Ang II was decreased or even abolished (depending on the concentration of candesartan used) when AT_1 receptors were blocked by candesartan. The results are in concordance with findings of another research group (Hadzhibozheva et al. 2013) which characterized the Ang II-induced contraction of the rat distal colon as mainly mediated by the AT_1 receptor. The effect of the AT_1 antagonist was similar between males and females, which is not in agreement with the previously referred alteration in the expression of AT_1 receptors induced by sex hormones (Paul et al. 2006; Komukai et al. 2010). Nevertheless, it is possible that the effect of sex hormone on receptor expression doesn't parallel the effect on receptor function. Interestingly, our results show that in the rat distal colon AT_2 receptors are functional and contribute to the net effect induced by Ang II. Namely by counteracting the constriction induced by stimulation of AT_1 receptors. This was evidenced since AT_2 blockade with PD123,319 increased the response to Ang II. It means that when AT_2 receptors are blocked, all exogenously applied Ang II is free to bind AT_1 receptors that cause contraction. This result opposes that of another research group (Hadzhibozheva et al. 2013) that reported AT_2-mediated Ang II-induced contraction of the rat colon. Ang II AT_2 receptors have also been implicated in the vasoconstrictive effect of Ang II in small mesenteric arteries of young spontaneously hypertensive rats (Touyz et al. 1999). However, the classical view of the RAS most usually associates the AT_2 receptor with relaxation of smooth muscle. In our experiment, the same strain of rat (Wistar Han) was used and the origin of drugs was the same as in the experiment of (Hadzhibozheva et al. 2013), the only difference was in the concentrations of used drugs. Hadzhibozheva et al. 2013 tested only the longitudinal colonic smooth muscle and in their experiments lower concentration of Ang II and higher concentration of PD123,319 was used.

8 Conclusions

The results of this work lead to the following conclusions:

- The reactivity of the circular smooth muscle of the rat distal colon is higher compared to longitudinal muscle. Although more reactive, the circular muscle seems to be less sensitive to contractile agents than the longitudinal muscle.

- The reactivity of the colonic longitudinal muscle to KCl, ACh and Ang II is similar between males and females. In the circular muscle, the reactivity to KCl and ACh is higher in females compared to males.

- In the longitudinal and circular muscle of the rat distal colon, the contractile response induced by Ang II is the balance between activation of AT_1 receptors, causing constriction, and AT_2 receptors, causing relaxation. Exception to this, in the colonic circular muscle of female rats, the response to exogenously applied Ang II is not mediated by AT_2 receptors.

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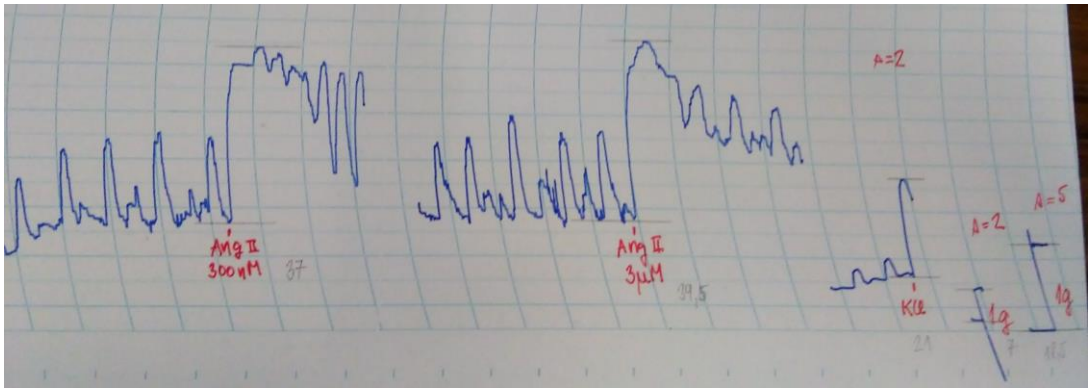
Appendix 1

Concentration of drug	Needed volume	Concentration in the organ bath
10^{-7} M	20 µl	100 pM
	60 µl	300 pM
10^{-6} M	20 µl	1 nM
	60 µl	3 nM
10^{-5} M	20 µl	10 nM
	60 µl	30 nM
10^{-4} M	20 µl	100 nM
	60 µl	300 nM
10^{-3} M	20 µl	1 µM
	60 µl	3 µM
10^{-2} M	20 µl	10 µM
	60 µl	30 µM

The volume of the dilution, which was added to the organ bath, to obtain the wanted concentration of the drug.

Appendix 2



Recording of the polygraph

1g	_____	7 mm
x (g)	_____	38 mm

x = 38/7 ≈ 5,43

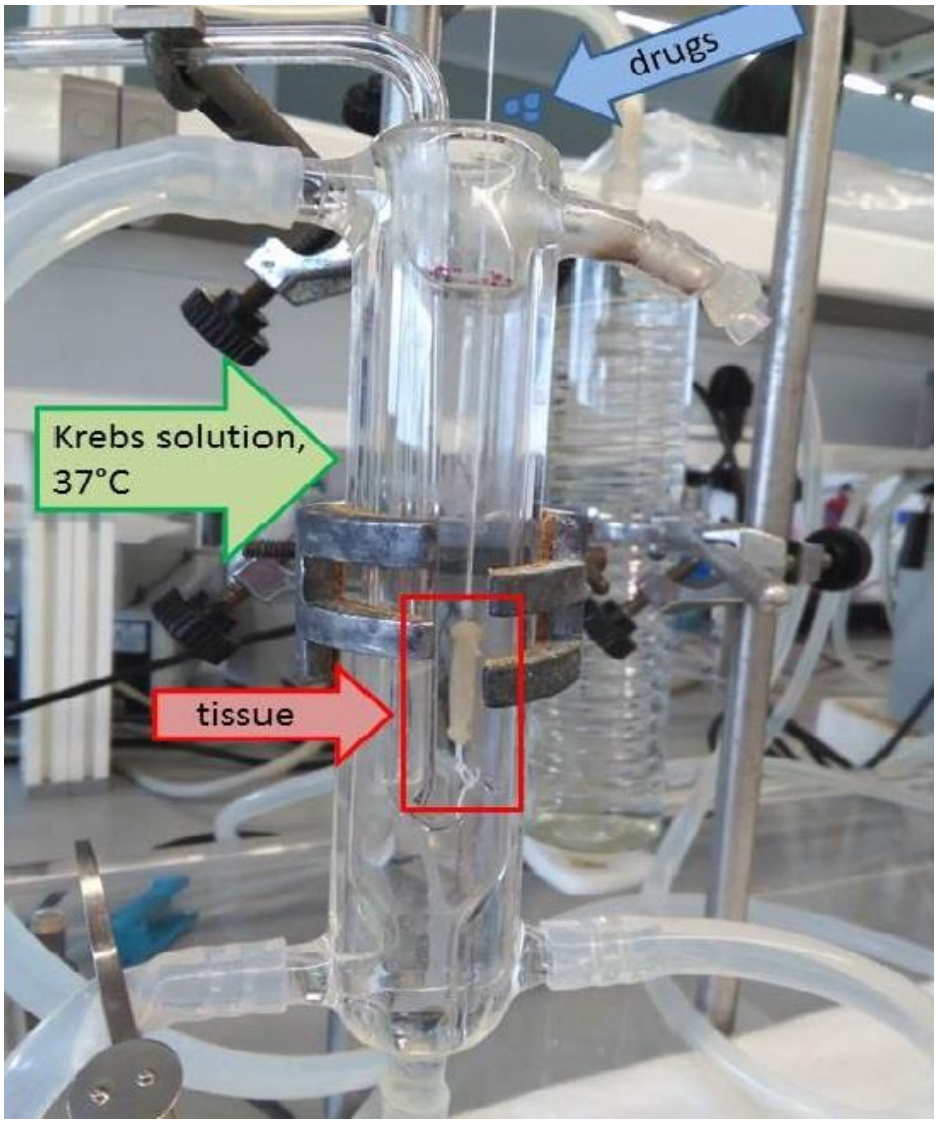
An example of calculation – transfer of the response from the millimetres to the grams

5,43 g	_____	26,67 mg
x (g)	_____	1000 mg

x = 5,43/26,67 × 1000 = 203,55 (g/g)

An example of calculation - evaluation of weight of the tissue

Appendix 3



Organ bath with the mounted tissue, filled with Krebs solution (37°C)