Effects of Transient Receptor Potential Ankyrin 1 (TRPA1) receptor agonists and the Roman chamomile on human and rodent isolated smooth muscle organ preparations

PhD-thesis

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Introduction

In the intramural ganglia (myenteric and submucous plexus) of the gastointestinal tract at least as many neurons take place as in the spinal cord (10^7-10^8 neurons). These neurons can be excitatory or inhibitory ones, in them they can stimulate or inhibit other neurons. On the other hand, enteric neurons relax or contract the circular or longitudinal smooth muscles (with the release of neuromuscular, neuroeffector transmitter molecules). These are called „enteric motoneurons". Their axons might be found in both muscular layers. Parallel with the effect of main mediators, co-mediators (co-transmitters) are also released. The presence of a modifying agent (modulator molecule) can also be significant regarding neurotransmitter mechanisms of the gut. Motoneurons innervate effector systems such as smooth muscle, pacemaker cells, blood vessels and mucosal glands.

The excitatory motoneurons release transmitters, that evoke contraction of muscles or the electrolyte and water secretion from the mucosa. The excitatory transmitters are acetylcholine and substance P (SP). Acetylcholine and VIP (Vasoactive Intestinal Peptide) stimulate the secretion from the intestinal crypts.

Inhibitory motoneurons decrease smooth muscle contractility. Their transmitters are ATP, VIP and mainly NO (nitric oxyde) **[see Furness et al. 2014].**

This complexity ensures the genesis of own (intrinsic) reflexes in the gastrointestinal tract (for example: the peristaltic reflex evoked by the tension of the gut wall).

This complex process and extent of neurons enables us to rank the enteric nervous system as a unique part of the autonomic nervous system **[see Furness et al. 1998].** The intrinsic neurons of the gall bladder and the bile ducts also belong to this system.

Altough the gut is able to function without extrinsic innervation, it stays under the influence of outer (extrinsic) nerves and even hormonal effects too *in vivo.* So, it is not surprising that a lot of molecules are playing a role in these broad-spectrum effects. The physiological/pathopysiological role of these presumed transmitters are proven in different systems and we must reckon with the differences in different animal species. (Of course, the propulsion and mixing of the intestinal content is implemented in every animal species, but the similarities in the function do not mean necessarily the same neurotransmitter background.)

The sensory neurons can also be classified as intrinsic and extrinsic ones. Intrinsic sensory neurons in the gut wall are able to detect the tension or other stimuli, they send the excitation through interneurons to motoneurons. By this way, they create the mechanism called „the law of the intestine", which means contraction of the circular muscle orally from the stimulus, and relaxation aborally **[see Mazet 2015].** Extrinsic sensory neurons (whose axons are arriving with vagal nerve or from the spinal cord) play an important role in sensations from the gut and autonomic reflex circuits.

The special feature of these afferent neurones is releasing transmitters in the periphery such as in the gastrointestinal tract. (This is also named „local efferent effect"). The function of the visceral organs might be affected by this; moreover, it offers quite a simple way for neurotransmitter identification **[see Barthó et al. 2004].** On the base of the revised Dale's principle, probably the same neurotransmitter combination is set free in both the central and peripheral nerve endings of the afferent neurons. That is why, the released transmitters are identical in the nerve endings of these extrinsic afferent neurons in the gut wall in the central nervous system **[see Barthó et al. 2008].** These sensory neurones can be stimulated e.g., with capsaicin, the main component responsible for the spiciness of hot pepper. Although many fibres of the vagal nerve are sensory (many of them capsaicin-sensitive), we know little about the molecules released from this nerve in the periphery.

We may also assume that the illnesses of the gastrointestinal tract affect the signal transduction mechanisms and the transmitter substances. It is hard to decide, whether these changes are the causes or the consequences of the disease. It is likely that, they take part in producig of the symptoms.

Nerve-muscle transmitters

As a lot of „transmitter candidates" can be shown with morphological tools in the enteric nervous system, we have to take the criteria seriously, which are used in the identification of transmitters and were developed in the first and second third of the 20th century.

The most important neurotransmitter criteria were and still are, as follows:

- the presence of the presumed substance

- the release of the transmitter due to nerve stimulation

- the effect of the administered –exogenous– „transmitter candidate" mimics closely the effect of the nerve stimulation, „identity of the effect"

- the specific antagonist of the suspected transmitter blocks both the effect of the exogenous agent and the effect of the nerve stimulation, „identity of antagonism"

If specific antagonist is not available, specific synthesis-inhibitor, receptor-desensitization, or knock-out animals might also be utilized

The responses –other than mediated by the „classical" neurotransmitters: acetylcholine and noradrenaline are also known as „non-adrenergic, non-cholinergic" (NANC) responses. The circumstances for NANC conditions can be reached e.g., by deploying atropine and an adrenergic neuron blocker agent. These responses may be excitatory or inhibitory ones **[see Barthó et al. 2005]**.

The topic of the TRP receptors is a hot-spot in scientific research under cellular and *in vivo* conditions. This issue is less frequently investigated in isolated organ laboratories in pharmacological research.

The above mentioned capsaicin-sensitive receptor is the TRPV1 receptor, whose gene was first cloned in 1997 **[Caterina, Julius et al. 1997].**

TRP receptors: short characterisation and classification

The TRP receptors are cation channels, which are present throughout the body. The abbreviation stands for: „transient receptor potential".

We differentiate TRPC, TRPV ("V", as vanilloid), TRPM ("M", as melastatin), TRPN, TRPA („A" as ankyrin) and TRPP, TRPML receptors too. In further classification, the subgroups are ranked with numbers (e.g., TRPM8).

They are sensitive for different sorts of chemical stimuli (e.g., piperine, capsaicin, menthol, allyl isothiocyanate); they might also be activated by heat, cold, tension, pressure, vibration. Many of them enhance intracellular calcium, when activated **[Caterina, Julius 2001; Vyklicky et al. 2008; Cavanaugh et al. 2008].**

The TRPV1 receptor: Capsaicin is the main component in hot pepper responsible for the hotness; it boosts the activation of the TRPV1 receptor. This receptor can be found in large numbers in sensory nerve endings. This causes the burning sensation and the erythema on the skin and mucosa **[see Barthó et al. 2004].** TRPV1 receptor is a cation channel, activated by high tempreture (> 43°C), low pH (pH <6.5) and different kinds of chemical substances such as: capsaicin, phorbol-esthers. Actually, it is a non-selective cation channel, whose activation leads to

Ca^{++} and Na^{+} influx (and in at least in part K^{+} efflux). The axonal membrane of the sensory nerve ending depolarises owing to these effects **[Yang et al. 2014].**

Resiniferatoxin (RTX) –which can be found in certain African *Euphorbia* species– is the most potent agonist of TRPV1 receptor– its affinity is 500 times higher than that of capsaicin. Piperine (which gives the spiciness of black pepper) stimulates in low concentration only the TRPV1 receptor, but in higher concentrations it activates the TRPA1 receptor too **[see Bencsik et al. 2015].**

TRPA1 receptor: This receptor shows massive co-localization with TRPV1 in capsaicin-sensitive afferent nerve endings **[Koivisto et al. 2014]**. Cold and allyl isothiocyanate activate it. Allyl isothiocyanate (AITC) gives the spicy taste and „strength" of mustard, wasabi and horseradish. Another activator of this receptor is cinnamaldehyde (CINN), which provides the fragrance of cinnamon **[Cavanaugh et al. 2008].**

TRPM8 receptor: The main activators of this receptor are low tempreture and menthol. Recently, icilin is also held as an activator of TRPM8 and this cation channel is also a suspected mechano-nociceptor **[see Xiaoyun et al. 2015].** An antagonist of this receptor and TRPV1 is the agent called BCTC **[see Benkó et al. 2012a].**

TRP receptors/ion channels are implicated in a rapidly growing number of functions in health and disease, such as visceral and somatic nociception, inflammation and inflammatory hyperalgesia, heat regulation and trophic effects **[Kaji et al. 2012, Bautista et al. 2013, Kaneko et al. 2014, Sousa-Valente et al. 2014, Koivisto et al. 2014, Kun et al. 2014, Benemei et al. 2015, Cenac et al. 2015, Mueller-Tribbensee et al. 2015, Mickle et al. 2015].**

AITC has an excitatory effect on the bowel movements of the mouse **[Capasso et al. 2012]** and of the guinea pig **[Barthó et al. 2013].** HC030031 is a selective and potent antagonist of TRPA1 **[Alexander et al. 2018].** AITC causes nerve-mediated, cholinergic contractions in the guinea pig small intestine, which is not hindered by HC030031, but surprisingly it is inhibited by the purinoceptor antagonist PPADS **[Barthó et al. 2013]**. Likewise, in the distal colon of mice AITC-evoked contraction was not hampered by the above mentioned TRPA1 antagonist **[Capasso et al. 2012]**.

There is as many as 28 kinds of TRP receptors in the mammalian gastrointestinal tract, which play role in tasting, chemo- and mechano-sensation, in the physiological functioning of the mucosa and in homeostasis too. The role of certain TRP receptors is highlighted in the Mg^{++} ion balance (TRPM6; TRPM7) or in the pacemaker activity of the intersitial cells of Cajal (TRPM7). The TRPM5 regulates the glucose-dependent insulin liberation from pancreatic β-cells, and it contributes to the taste-sensation too **[see Holzer 2011]**.

The possible role of serotonin in the gastrointestinal peristalsis.

Serotonin (5-hydroxytryptamine, 5-HT) is present in the gastrointestinal tract of mammals **[see Spiller 2007, Grundy 2008]**. While enterochromaffin cells both synthesize and release 5-HT, enteric neurons take it up from their environment and release it in response to appropriate stimuli. Intestinal smooth muscle cells are directly contracted by 5-HT in many preparations **[Prins et al. 1997, Gelal et al. 1998, Delesalle at al. 2006]**, but in the guinea pig small intestine, both a muscular and a more powerful, neuronal effect is present, which made pioneering researchers propose the presence of M (morphine-sensitive) and D (dibenzyline /phenoxybenzamine/ -sensitive) receptors **[Gaddum at al. 1957]**. Thus, the former one may mediate neural (mostly cholinergic **[Day at al. 1963, Brownlee at al. 1963]**) and a latter one the direct muscular component of the response. The direct smooth muscle contracting effect was found inhibited by 10 μg/ml of the 5-HT antagonist methysergide **[Costa, Furness 1979]**. The 5-HT-evoked neural contractile responses in the guinea pig ileum seem to be little affected by methysergide, and are probably mediated by 5-HT_3 and 5-HT_4 receptors located at cholinergic neurons, of which the 5-HT_3 is an ionotropic (cation channel) and the 5-HT_4 a G_s-protein-coupled receptor **[see Alexander et al. 2018]**. (To date, 7 types /$5\text{-HT}_{1–7}$/ of serotonin receptors have been discovered, with multiple isoforms existing within a group.) The musculotropic 5-HT receptors in the guinea pig ileum, at least partly, belong to the 5-HT_1 and 5-HT_2 type **[Eglen at al. 1990]**.

The sensory-stimulant AITC is an agonist of TRPA1 receptor **[Alexander et al. 2018]**. Probably, the sites of the effect of AITC are not confined only to capsaicin-sensitive afferent nerve endings or solely to the TRPA1 channel, regarding the mice's colon and guinea pig ileum **[Capasso et al. 2012; Barthó et al. 2013]**. Morover, Nozawa et al. (2009) presumed that serotonin –released from the enterochromaffin cells, is responsible for the AITC-evoked excitatory action in the guinea pig ileum.

Of the many TRP receptors, this thesis is dealing with the TRPV1, TRPA1, and TRPM8 receptor due to their significance in practice in the gastrointestinal tract. We applied specific antagonists of these receptors, and capsaicin desensitisation –whose effect implies the total loss of function of the capsaicin-sensitive afferent nerve ending **[see Barthó et al. 2004]**.

Brief characterization of Roman chamomile

The Roman chamomile (Fig. 1.) (*Chamaemelum nobile, Anthemis nobilis*) is a medicinal plant and belongs to the *Asteraceae* family **[EMA-HMPC, 2012].**

Fig. 1. Roman chamomile (Köhler et al. 1898)

The herb is utilized either in raw or dried form. The tea of the plant or the hydroethanolic extract of it might be used against mild gastrointestinal complaints, such as bloating or bowel cramps **[EMA-HMPC, 2012].** Roman chamomile also is believed to possess anti-inflammatory **[Baghalian et al. 2011],** smooth-muscle relaxant **[European Pharmacopoeia, 2008; British Herbal Pharmacopoeia, 1971],** antihypertensive **[Zeggwagh et al. 2009],** antibacterial activity **[Piccaglia et al. 1993, Chao et al. 2000, Bail et al. 2009]**.

Our goal was to demonstrate or refute the smooth muscle relaxing effect of the herb **[Kandelous et al. 2016, Augustin et al. 1948, Rápóti and Romváry 1974, Melegari et al. 1988, Rossi et al. 1988, Bradley et al. 1992]** in isolated organ experiments.

The smooth muscle preparations included human jejunal specimens, guinea pig ileum, urinary bladder and rat colon, ileum, gastric fundus.

Aims

Our research group investigated the effects of AITC, CINN and Roman chamomile in the isolated smooth mucle organs –including: small and large bowel, urinary bladder, trachea.

We applied –for obtaining contractile or relaxant response– the TRPA1 agonist AITC and CINN or the alcoholic extract (with 70% ethanol) of Roman chamomile (RKE), the essential oil of the herb, or the fractions and flavonoids of this plant.

Moreover, we often used electical field stimulation of the nerves for making the smooth muscle organs exert contractile response.

We have also seized the opportunity to examine the human small intestine preparations under similar circumstances, owing to the cooperation with the Department of Surgery at our University Clinical Center.

Due to our collaborations with the Department of Pharmacognosy University of Szeged, we could gain the hydroethanolic extract (RKE) of this medicinal plant and the essential oil, fractions and flavonoids of it, too.

We aimed to examine the pharmacological background of the issues below. Our questions to be answered were the followings:

1./a. What is the mechanism of action in the contractile effect of AITC in human longitudinal and circular jejunal smooth muscle preparations? Is there any difference in the receptorial background in the contractile action of AITC, compared to the guinea pig small bowel?

1./b. What is the pharmacological background in the contractile and relaxant response evoked by AITC in the guinea pig ileum? Have serotonin or the mucosa really a role in the contractile action of AITC?

1./c. What is the mechanism of the effects of AITC in other organs of the guinea pig, is there a TRPA1 receptor-independent mechanism in the processes?

1./d. What are the mechanisms of action of CINN, (contractile and relaxant effects) in different guinea pig smooth muscle preparations? Do TRPA1 antagonists inhibit the responses in smooth muscle activity caused by CINN? What is the effective concentration of these antagonist, still not evoking non-specific effects? How does CINN affect human smooth muscle preparations?

2./a. Is there exact evidence of the relaxant effect of Roman chamomile? What are the effects and their mechanisms of the plant's RKE, essential oil, fractions and flavonoids in rodent and human smooth muscle organs?

2./b. What kind of components of the plant are responsible for the spasmolytic effect of RKE? What is the pharmacological background of the essential oil's relaxant action, what sort of concentration-response relations can be revealed in this inhibitory action? Do TRP receptors or axonal conduction play a role in this process?

Materials and methods

Experimental models

Our isolated organ experiments were performed under isotonic conditions and with auxotonic (near-isotonic) systems **[see Barthó, Sándor et al. 2014].**

Human jejunal segments were obtained from the operation theatre. Informed consent was gained from the patients, and the experiments were approved by both the National and Regional Ethical Committees. The experiments were carried out on human jejunal longitudinal preparations (2 mm x 30 mm, with the mucosa-submucosa removed with fine scissors). Some circularly oriented preparations were also used. The strips were put into jacketed organ baths containing oxygenated Krebs-Henseleit solution, kept at 37°C. Movements of the tissues were recorded isotonically, by means of lever transducers and bridge amplifiers. The load on the tissues was 10 mN. We used ink-writers or personal computers for recording. The organ baths were fitted with a pair of platinum electrodes (at the top and the bottom), 4 cm apart, for electrical field stimulation (voltage: 15 V/cm; pulse width: 0.1 ms; „trains" of 2 Hz for 20 s). After an equilibration period of 40 min., maximal spasm was evoked by acethylcholine (30 μM given for 3 min., followed by 30 min. of rest).

All experiments on animal tissues have been approved by the Regional Committee for Animal Research. The animals were kept under the following circumstances: moisture of the air: 55±10 %, tempreture: 22±2 °C, dark/light cycle: 12/12h, replacement of air: 15-fold per hour. The animals received standard rodent-chow and tap water *ad libitum*.

Guinea pigs (short-haired, coloured) of either sex, weighing 330–450 g were stunned by a blow to the occiput and bled out. Whole segments of the **ileum** or distal **colon** (approximately 2 cm in length) were suspended in organ baths containing oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit solution at 37 °C. Composition of the bathing solution was as follows (mmol/l), NaCl 119, $NaHCO_3$ 25, KCl 2.5; $MgSO_4$ 1.5; $CaCl_2$ 2.5; KH_2PO_4 1.2; glucose 11. Longitudinal movements were recorded isotonically, using Hugo Sachs-Harvard Instruments transducers and bridge amplifiers. The load on the tissues was 7 mN. Experiments commenced after an equilibration period of 40 min, after which the maximal longitudinal spasm was evoked by histamine (3 μmol/l for 30 s). In part of the

experiments, ileal „strip” preparations containing the longitudinal muscle and myenteric plexus were used. The load on the tissues was 3 mN **[Sándor at al. 2016b].** In some experiments on the isolated ileum of the guinea pig, electrical field stimulation with single shocks was applied through a pair of platinum electrodes (situated at the top and bottom of the organ bath; 4 cm apart), for eliciting cholinergic „twitch” responses. Parameters of stimulation were near-maximal voltage of 15 V/cm; 0.1 ms pulse width; 0.05 Hz **[see Benkó et al. 2012b].** Stimuli were delivered by an EXP-ST 01 stimulator (Experimetria, Budapest, Hungary), completed by a power amplifier (Ulrich Design, Pécs, Hungary).

In the case of guinea pig **urinary bladder**, the experimental protocol was as follows **[Benkó et al. 2012a]**: During preparation the bladder was put into Krebs-Henseleit solution in a Petri dish. We divided the bladder in saggital direction, so we recieved two halves, then we made a longitudinal detrusor-muscle preparation of each half by an incision. The load on the tissue was 5 mN. We evoked the maximal contracion with 100 mmol/l KCl at the end of the experiment.

In the case of guinea pig **trachea**, the preparation method was the following **[Szolcsányi, Barthó 1982]**: During preparation the trachea was put into Krebs-Henseleit solution in a Petri dish. The tracheal cartilages were cut into two halves in longitudinal direction (the smooth muscle remained unscathed) and we made „zig-zag” preparations consisting of 5 to 7 cartilage rings. The load on the tissue was 3 mN. We evoked the maximal contracion with 100 mmol/l KCl at the end of the experiment.

The ileal preparation protocol was applied in the case of the **esophagus** too **[see in details: Barthó, Lénárd at al. 1999]**. The load on the tissue was 10 mN. We evoked the maximal contracion with 200 mmol/l KCl at the end of the experiment.

We dissected 2 cm longitudinal preparations from the guinea pig **gastric fundus**. The load on the tissue was 5 mN. We evoked the maximal contracion with 200 mmol/l KCl at the end of the experiment.

In the case of **taenia coeci**, we used the taenia libera. We obtained preparations 15-20 mm in lenght **[see in details: Lénárd et al. 2000]**. The load on the tissue was 5 mN. We evoked the maximal relaxation with 10 μmol/l isoprenaline at the end of the experiment.

Female and male **Wistar rats** weighing 220–330 g were stunned by a blow to the occiput and bled out from the carotid arteries. Whole segments of the guinea pig

ileum and distal colon (approximately 2 cm in length) were set up as preparations. Rat stomachs were opened along the lesser and greater curvatures and the two halves were cut into longitudinally-oriented strips of approximately 2 cm in length. All preparations were suspended in organ baths containing oxygenated (95% O_2, 5% CO_2) Krebs–Henseleit solution at 37 °C. Movements of the tissues were recorded isotonically, by means of lever transducers and bridge amplifiers (Hugo Sachs –Harvard Apparatus, March– Hugstetten, Germany). The load on the tissues was 7 mN (rat small and large intestine) or 5 mN (rat fundus strip) **[see Benkó at al. 2012b].**

The experiments commenced after an equilibration period of 40 min (rat ileum and distal colon) or 75 min (rat stomach). Maximal spasm was evoked by acethylcholine (30 μM given for 3 min., followed by 30 min. of rest). In the rat ileal preparation, electrical „train" stimuli causing approximately half-maximal contraction were evoked by electrical field stimulation (near-maximal voltage of 15 V/cm; 0.1 ms pulse width; multiple electrical shocks at 4 Hz for 30 seconds), applied by means of a high-performance stimulator (Experimetria, Budapest, Hungary), through a pair of platinum wire electrodes, placed at the top and the bottom of the organ bath –4 cm apart; as previously.

The process of α,β-methylene ATP (α,β-metATP) desensitization was the following: the agent was given into the organ bath in a concentration of 15 μmol/l for 10 minutes, then *plus* 15 μmol/l concentration was administered for 15 minutes. Actually, the second administration was ineffective. The investigated substances were given into the bath in the presence of α,β-metATP **[Benkó et al. 2005].** (α,β-metATP is the metabolically more stable form of ATP, which is activating and desensitizing mainly P_{2x} receptors.)

Capsaicin tachyphylaxis was carried out by administering capsaicin to the bath in 10 μmol/l concentration for 10 minutes. After washing out, 1 hour resting period commenced with multiple rinsing **[see Barthó et al. 2004].**

One of our aims was to find a serotonin antagonist combination or procedure which is able to inhibit the effect of exogenous 5-HT in the smooth muscle, without non-specific effects. We applied the substance called SB204070 for blocking 5-HT_4 receptors (2 μmol/l) **[Alexander et al. 2018],** Y25130 (azasetron, 1 μmol/l) was deployed to inhibit 5-HT_3 receptors **[Sato et al. 1992].** Methysergide (0.3 μmol/l) was administered as a broad-spectrum serotonin receptor antagonist **[Prins et al. 1997, Mylecharane 1989].**

Results were provided in relative values, where 100% contraction means maximal spasm of the preparations from the basal tone and, in precontracted preparations, 100% relaxation means relaxation of the preparation to the level before adding the precontracting agent, in the case of the organs with low basal tone. In other cases, the relaxation was compared to the maximum inhibitory response evoked by 10 μmol/l isoprenaline.

Drugs

Cinnamaldehyde /CINN/, allyl isothiocyanate /AITC/ (both from Sigma); α,β-methylene ATP /α,β-metATP/ (Tocris), atropine hydrochlorid (Sigma), PPADS (pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid tetrasodium salt) (Tocris), tetrodotoxin [TTX] (Tocris), capsaicin (Ashian Herbex Ltd.), indomethacin (Sigma), acetylcholine (Sigma), serotonin (Tocris), histamine (Sigma), propranolol hydrochlorid (Sigma), suramin (Sigma), apamin (Sigma), TEA {tetraethyl-ammonium chloride} (Sigma). L-NOA {N^G-nitro-L-arginine}: (Sigma), A967079 ((1*E*,3*E*)-1-(4-Fluorophenyl)-2-methyl-1-pentene-3-one oxime) (Tocris), HC030031 (2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7*H*-purin-7-yl)-*N*-(4-isopropylphenyl) acetamide) (Tocris), BCTC (4-(3-Chloro-2-pyridinyl)-*N*-[4-(1,1-dimethylethyl)phenyl]-1-piperazinecarboxamide) (Biomol). SR140333 (1-[2-[(3*S*)-3-(3,4-Dichlorophenyl)-1-[2-[3-(1-methylethoxy)phenyl]acetyl]-3-piperidinyl]ethyl]-4-phenyl-1-azoniabicyclo [2.2.2] octane chloride) (Sanofi), SR48968 (N-[(2S)-4-(4-acetamido-4-phenylpiperidin-1-yl)-2-(3,4-dichlorophenyl)butyl]-N-methylbenzamide) (Sanofi), SR142801 (N-[1-[3-[(3R)-1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl]propyl]-4-phenylpiperidine-4-yl]-N-methylacetamide) (Sanofi), isoprenaline (Sigma), glibenclamide (Sigma), methysergide (Sandoz), SB204070 (8-Amino-7-chloro-2,3-dihydro-1,4-benzodioxan-5-carboxylic acid, 1'-butyl-4'-piperidinylmethyl ester) (Tocris), Y25130 (*N*-(1-Azabicyclo [2.2.2] oct-3-yl)-6-chloro-4-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazine-8-carboxamide hydrochloride) (Tocris), prostaglandin $F_{2\alpha}$ tris salt (Sigma).

The solvent for CINN and AITC was Krebs-Henseleit solution. Atropine, tetrodotoxin, histamine, serotonin, apamin, suramin, propranolol; α,β-metATP, acetylcholine; PPADS, TEA, L-NOA, Y25130, methysergide and isoprenaline were dissolved in physiological saline. Indomethacin, prostaglandin $F_{2\alpha}$ /$PGF_{2\alpha}$/ and capsaicin were dissolved in pure ethanol. The solvent was dimethyl sulfoxide (DMSO) for BCTC and A967079, HC030031; SR140333, SR48968, SR142801, glibenclamide, SB204070.

Drugs used in the tests with Roman chamomile

We purchased the essential oil from „Aromax" Company, all other materials derived from the Institute of Pharmacognosy, Department of Pharmacology University of Szeged. **The applied extraction and refinery processes are beyond the scope of this thesis.**

For the pharmacological experiments, the plant's hydroethanolic extract was prepared according to the description of the European Medicines Agency monograph (EMA-HMPC, 2012). Ten grams of plant material was extracted with 70% ethanol (3×100 ml) via ultrasonic bath, evaporated in vacuum and lyophilized (yielded 3.169 %). Part of the Roman chamomile hydroethanolic extract was fractionated to obtain herbal extract fractions with different compositions for further experiments. Vacuum liquid chromatography on polyamide with elution by methanol-H_2O (20:80, 40:60, 60:40, 80:20, 100:0) was used to obtain fractions from the hydroethanolic extract as follows: F20, F40, F60, F80, and F100; respectively. We solved the lyophilized substances in DMSO, so we made stock solutions in a concentration of 200 mg/ml as a first step, then we diluted these further as needed.

Based on their retention times and mass spectrometric data, methallyl angelate, 3-methyl pentyl angelate, 2-methylbutyl angelate and 3-methylamyl isobutyrate were identified as the major constituents of the herb's essential oil (see in details: Sándor, Mottaghipisheh et al. 2018) by the Department of Pharmacology University of Szeged. The RKE's major components were the flavonoids, such as: eupafolin, luteolin, hispidulin and apigenin **[Sándor, Mottaghipisheh et al. 2018].**

Further reagents: α,β-metATP (Tocris), atropine (Sigma), prostaglandin $F_{2\alpha}$ tris salt (Sigma), PPADS (Tocris), tetrodotoxin (Tocris). Capsaicin (Ashian Herbex Ltd.), indomethacin (Sigma), acetylcholine (Sigma), histamine (Sigma), propranolol (Sigma), methysergide (Sandoz), SB204070 (Tocris), Y25130 (Tocris), isoprenaline (Sigma).

The solvent in the case of atropine, tetrodotoxin, Y25130, histamine, propranolol, α,β-metATP, acetylcholine, PPADS, methysergide and isoprenaline was physiologic saline. $PGF_{2\alpha,}$ indomethacin; and capsaicin were dissolved in pure ethanol. The solvent for SB204070 was DMSO.

Statistical analysis

Quantitative results are given as mean ± SEM. At most 2 preparations from the same animal were used for each type of experiment. The Mann–Whitney U test was used for the comparisons of two independent groups; more than two independent samples were compared by the Dunn test: the post-test of Kruskal–Wallis test; two paired samples were compared by the Wilcoxon-signed rank test. The statistical program GraphPad Prism 5 was used throughout. A probability of $P < 0.05$ or less indicated statistical significance.

Results

I./a, AITC-induced contractile effect in human small intestine:

AITC caused tonic contraction on the human jejunal longitudinal preparations. As the effect tended to decline upon repeated administrations, the preparations were arranged in independent groups and AITC was administered only once to each preparation. The response showed concentration dependence in the range of 100 and 600 μmol/l. The concentration of 300 μmol/l of AITC was chosen for pharmacological analysis. The effect was not inhibited by tetrodotoxin (0.5 μmol/l), but, surprisingly, nearly abolished by atropine (0.5 μmol/l). This finding was confirmed with two low concentrations of scopolamine, another muscarinic receptor antagonist; 30 nmol/l and 0.1 μmol/l of scopolamine caused significant, 68 and 100% reduction, respectively. The effect of AITC was significantly enhanced by the cholinesterase inhibitor physostigmine (50 nM for 15–20 min.); an enhancement of 43% was found, as compared with control preparations taken from the same individuals. A combination of the purinoceptor antagonists PPADS (50 μmol/l) and suramin (100 μmol/l) failed to influence the effect of AITC, but the TRPA1 antagonists HC030031 or A967079 caused significant reduction at 30 μmol/l and 1 μmol/l, respectively. The solvent of HC030031 (DMSO) had no inhibitory effect on the AITC response. Pre-treatments that had no obvious influence on the effect of AITC included capsaicin desensitization (capsaicin 10 μmol/l added for 10 min., followed by 50 min. of washout), the TRPV1/TRPM8 antagonist BCTC **[Benkó et al. 2012a]** (2 μmol/l), the ganglion blocker hexamethonium (100 μmol/l) or indomethacin (5 μmol/l).

I./b, AITC-induced contractile effect in guinea pig ilea:

Experiments commenced after an equilibration period of 40 min, after which the maximal longitudinal spasm was evoked by histamine. After multiple washout,

the contractile effects of 5-HT or the putative TRPA1 stimulant AITC were tested. 5-HT was added to the bathing fluid for 20 s twice, 40 min apart (another renewal of the bath solution was performed at min. 20th). A single concentration of 5-HT (0.5-2 μmol/l) was used that evoked a response of approximately 60% of the maximal contraction. The responses were found to be reproducible. Correction of the 5-HT concentration, if necessary, was performed after the first administration of 5-HT. Thus, 5-HT was administered twice to most and 3 times to some preparations. During the control exposure, the solvent of the drug tested on the 5-HT response was present in the organ bath (lack of inhibitory effects of the solvents was also assessed in separate experiments). The contact time of the antagonist drugs tested was 20 min. 5-HT desensitization was achieved by administering 5+5 μmol/l of the drug, 5 min apart. Ten min after the second exposure (without washing), the preparations were rendered fully unresponsive to an addition of a smaller amount of 5-HT (0.5-2 μmol/l) that evoked approximately half-maximal contraction before desensitization. The contact time for AITC was 2 min; during this period, its contractile actions fully developed and also faded away. In part of the experiments, strip preparations containing the longitudinal muscle-myenteric plexus (LM-MP) **[Paton és Vizi 1969]** were used. The load on the tissue was 3 mN. In some experiments, electrical field stimulation with single shocks was applied through a pair of platinum electrodes (situated at the top and bottom of the organ bath), for eliciting cholinergic „twitch" responses. Parameters of stimulation were near-maximal voltage of 15 V/cm 0.1 ms pulse width, 0.03 Hz. Stimuli were delivered by an EXP-ST 01 stimulator (Experimetria, Budapest, Hungary), completed by a power amplifier (Ulrich Design, Pécs, Hungary). The drug under test was administered during continuous stimulation. These responses were fully sensitive to tetrodotoxin (0.5 μmol/l).

Sub-maximal responses to **serotonin** were reproducible under the circumstances of this study (both without and in the presence of DMSO); DMSO itself had no consistent effect. The contractile action of serotonin was moderately but significantly reduced by methysergide (0.3 μmol/l), SB204070 (2 μmol/l) or Y25130 (1 μmol/l). A nearly full inhibition of the 5-HT response was found with a combination of all 3 antagonists in these concentrations. The combination of three 5-HT receptor antagonists abolished the contractile effect of 5-HT in strip preparations as well. 5-HT desensitization caused a full inhibition of serotonin-evoked half-maximal responses both in the whole ileum and in LM-MP strip preparations.

On the contrary; as we reported earlier, **AITC** caused transient contraction on the ileum. The present experiments also show that a similar response is also elicited by AITC on the LM-MP preparation. The concentration chosen for pharmacological analysis (200 μmol/l AITC) was selected on the basis of previous experiments and preliminary experiments on the LM-MP preparation (current study). The excitatory effect of AITC (200 μmol/l) was **not influenced** by a combination of three 5-HT antagonists or by 5-HT desensitization either in the whole ileum or in LM-MP preparations. Similarly to the whole ileum, tetrodotoxin or atropine (0.5 μmol/l each) abolished the contractile effect of AITC (200 μmol/l) on LM-MP preparations. The purinoceptor antagonist PPADS (50 μmol/l) showed a profound inhibitory effect **[see Barthó et al. 2013]** in LM-MP strips as well.

I./c, AITC-induced contractile effect in guinea pig esophagus:

The selected concentration of AITC was 1 mmol/l, this resulted sub-maximal contraction (every smooth muscle preparation recieved AITC only once). TTX reduced the excitatory response significantly, by 44%. On the other hand atropine did not decrease the contractions. For evaluation of the participation of tachykinins, we applied specific tachykinin NK receptor blockers; such as SR140333 for blocking tachykinin NK_1 receptors, SR48968 for blocking NK_2 receptors, SR142801 for the inhibition of NK_3 receptors. The 3 tachykinin-antagonist (SR140333 3 μmol/l, SR48968 3 μmol/l and SR142801 0,1 μmol/l) **[Barthó, Lénárd et al. 1999]** diminished the contractions by 63%, (significantly in comparison with the DMSO control). The TRPA1-antagonist, A967079 (1 μmol/l) did not cause any significant difference comparing to its DMSO control.

I./d, AITC-induced contractile effect in guinea pig urinary bladder:

We chose 200 μmol/l concentration of AITC. Indomethacin caused significant inhibition, by 37%. On the other hand, the TRPA1 antagonist (A967079; 1 μmol/l) resulted significant inhibition of the contractile response (44% inhibition); also without non-specific effects.

I./e, AITC-induced contractile effect in guinea pig trachea:

AITC's dose was 200 μmol/l, only capsaicin tachyphylaxis caused significant, 90% reduction in the contractile responses. The muscarinic receptor blocker atropine, voltage-gated Na^+ channel blocker TTX; the TRPA1 antagonist HC030031, the TRPM8/V1 specific inhibitor BCTC, the purinergic receptor

blocker PPADS and the non-selective cyclooxygenase blocker indomethacin were without effect.

I./f, AITC-induced contractile effect in guinea pig gastric fundus:

The elected concentration of AITC was also 200 μmol/l, this elicited sub-maximal contractions. Regarding the reproducibility; the repeated administration yielded decreased contraction, after 40 min. of incubation period and multiple rinsing. So, AITC was given only once to gastric fundus. Atropine (0.5 μmol/l) decreased the contractions by 69 %; so did the P_2-purinoceptor blocker PPADS by 68 % (both significant). On the other hand, TTX (0.5 μmol/l) was ineffective.

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I./g, AITC-induced relaxant effect in guinea pig ilea:

On precontracted preparations (histamine-precontraction with approximately half-maximal plateau, in the presence of atropine and TTX), AITC (for 10 min.) evoked concentration-dependent relaxation. 200 μmol/l AITC caused approximately half-maximal relaxation. The relaxing effect of 200 μmol/l AITC was not reproducible and was not influenced by apamin (1 μmol/l), glibenclamide (10 μmol/l) or A967079 (1 μmol/l) and HC030031 (30 μmol/l). Propranolol (1 μmol/l) had a mild and insignificant inhibitory effect. TEA (1 mmol/l) moderately, but significantly reduced the effect of AITC. Higher concentrations of TEA could not be used because of a contractile effect. Capsaicin desensitization failed to influence the relaxant effect of AITC, as compared to solvent- and time-matching controls **[see Sándor et al. 2019].**

For evaluating the pharmacological background of these relaxations we used apamin –a component of bee venom– to block the small conductance Ca^{++} activated K^+ channels (1 μmol/l) **[Vergara et al. 1998]**. We applied glibenclamide (10 μmol/l) **[Sun et al. 1994, Fujimoto et al. 2006]** for the inhibition of ATP-dependent K^+ channels. TEA (tetraethyl-ammonium chloride) 1 mmol/l **[Suarez-Kurz et al. 1991]** was deployed for blocking the voltage-gated K^+ channels and non-selectively blocking the Ca^{++} activated K^+ channels **[Alexander et al. 2018]** on neural elements and on smooth muscle too **[Fan et al. 1994].**

I./h, AITC-induced relaxant effect in guinea pig colon:

Owing to the high intrinsic tone of the distal colon no precontracion was needed. 200 μmol/l AITC caused sub-maximal relaxations. These inhibitory responses were not reproducible; for this reason, independent groups were used. Apamin caused mild, but significant inhibition. Indomethacin yielded 15% decrease,

capsaicin desensibilisation resulted in 12% decrease in comparison with its alcohol solvent control; these changes proved to be significant. TTX caused the strongest inhibition, with 39%; which was also statistically significant.

I./i, AITC-induced relaxant effect in guinea pig taenia coeci:

The concentration of AITC was 200 μmol/l; this elicited the two-third of the maximal relaxation. This inhibitory answer was not repeatable, so AITC was administered only once for taenia. The non-selective potassium channel blocker TEA or TTX resulted in significant lessening in relaxations. Tetraethyl-ammonium elicited a vast 71% diminishing in relaxation.

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I./j, CINN-induced contractile effect in guinea pig ilea:

CINN (200 μmol/l for 3 min.) caused quick and transient contraction. The contraction was reproducible after an interval of 40 min. Thus, the peak amplitudes of the contraction in response to the first and second administrations were compared in a pharmacological analysis, where a standard concentration of 200 μmol/l of CINN was used. Atropine, TTX (both at 0.5 μmol/l) or the broad-spectrum purinoceptor antagonists PPADS (50 μmol/l) strongly inhibited or abolished the CINN-induced contraction. Also, the broad-spectrum purinoceptor antagonist suramin (100 μmol/l) caused a significant, 62% inhibition. The α,β-metATP desensitisation resulted 90% depression. The TRPA1 receptor/channel blocker A967079 (1 μmol/l) and HC030031 (30 μmol/l) significantly reduced the contractile response, by 60% and 76%; respectively **[Sándor et al. 2019]**.

I./k, CINN-induced contractile effect in guinea pig esophagus:

The reproducibility of the second CINN administration was not reliable due to the elevation of the basal tone; so we used independent groups of preparations. CINN (1 mmol/l) caused sub-maximal (range: 50-70 %) excitatory response on esophagus. Atropine or TTX (0.5-0.5 μmol/l) did not lessen the contractions; on the other hand, capsaicin tachyphylaxis abolished the CINN-evoked excitatory responses **[Sándor et al. 2019]**.

I./l, CINN-induced contractile effect in guinea pig urinary bladder:

These responses did not prove to be reproducible; therefore, independent groups of preparations had to be compared. The contractile action of 200 μmol/l CINN was not influenced by atropine, TTX, PPADS, α,β-metATP desensitization (a standard procedure for inhibiting purinergic neurogenic contractions in the bladder) or A967079 and HC030031. Contraction in response to CINN was

strongly (with 70%) reduced by capsaicin desensitization. The TRPV1 antagonist BCTC did not influence the contractile action of CINN **[Sándor et al. 2019].**

I./m, CINN-induced contractile effect in guinea pig trachea:

The CINN concentration chosen for the experiment was 200 μmol/l. The muscarinic blocking agent atropine decreased the tracheal-contractions to its quarter, and capsaicin tachyphylaxis hampered the contractions, by 88% **[Sándor et al. 2019]**. The voltage-gated Na^+ blocker TTX, TRPM8 and -V1 antagonist BCTC; and the purinergic receptor blocker PPADS were not effective in inhibiting of the CINN-evoked contractions of guinea pig trachea.

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I./n, CINN-induced relaxant effect in guinea pig ilea:

On precontracted preparations (histamine-precontraction with approximately half-maximal plateau, in the presence of atropine and TTX), CINN (30–200 μmol/l for 10 min.) evoked concentration-dependent, reproducible relaxation. 100 μmol/l CINN caused approximately half-maximal relaxation. The relaxing effect of 100 μmol/l CINN was reproducible after an interval of 40 min, and was not influenced by propranolol (1 μmol/l), apamin (1 μmol/l), glibenclamide (10 μmol/l) or A967079 (1 μmol/l). TEA (1 mmol/l) moderately (with 21%); but significantly reduced the effect of CINN. Higher concentrations of TEA could not be used because of a contractile effect. Capsaicin desensitization failed to influence the relaxant effect of CINN, as compared to solvent- and time-matching controls **[Sándor et al. 2019].**

I./o, CINN-induced relaxant effect in guinea pig colon:

The concentracion used was 200 μmol/l CINN. The maximal relaxation was elicited by isoplenaline (10 μmol/l) at the end of the experiment. Due to the unreproducible relaxing responses, we administered CINN only once. Neither TTX, nor PPADS, nor apamine inhibited the relaxing responses.

I./p, CINN-induced relaxant effect in guinea pig urinary bladder:

Relaxation induced by CINN (200 μmol/l) on the histamine-precontracted bladder strip was not significantly influenced by capsaicin pretreatment (solvent- and time-matching control, versus capsaicin-treated relaxation) **[Sándor et al. 2019].** The inhibitory responses for CINN were not reproducible, so that independent groups of preparations had to be used. Neither TTX, nor apamin elicited decrease in the inhibitory responses to CINN.

I./q, CINN-induced relaxant effect in human small bowel:

CINN (200 μmol/l) exerted relaxant resposes to human smooth muscle preparations. Lower concentrations than this were not effective. We used $PGF_{2\alpha}$ (1 μmol/l) for achieving precontracion state –as this organ posesses low basal tone. (The sub-maximal response was compared to acethylcholine maximum – added at the beginning of the experiment. The $PGF_{2\alpha}$ contact time was 20 min., until we obtained the plateau-phase. In these precontracted preparations, 100% relaxation meant relaxation of the preparation to the level before adding the precontracting agent.) The reproducibility was not complete, so CINN was administered only once. On human jejunal pecontracted smooth muscle preparations TTX, TEA, capsaicin desensibilization or A967079 (1 μmol/l) did not diminish the CINN-evoked relaxation; neither in longitudinally-, nor in circularly-oriented smooth muscle preparations.

I./r, CINN-induced relaxant effect in guinea pig taenia caeci:

CINN caused on the guinea pig taenia caeci concentration-dependent relaxations, and in this case; precontraction was also not necessary.

II. The experiments have been carried out with Roman chamomile, which has been used traditionally for the symptomatic treatment of mild, spasmodic and other gastrointestinal complaints, however, the presumed smooth muscle-relaxant effect has not been confirmed experimentally in the literature. The aim of our study was to investigate the effect of an extract of the plant (RKE), its fractions and the essential oil of the plant, as well as of four of its flavonoid components.

The phytochemical analysis of the extract revealed the presence of flavonoids in the plant, four of which (apigenin, luteolin, eupafolin, and hispidulin) were isolated and identified in the Department of Pharmacology University of Szeged **[Sándor, Mottaghipisheh et al. 2018]**. RKE was fractionated on polyamide to gain fractions with different flavonoid content in order to examine the role of these compounds in the effect on smooth muscles. The flavonoid content of the extract and fractions was analyzed by HPLC. Pharmacological experiments were carried out with RKE, its fractions (F20, F40, F60, F80, and F100), four flavonoids (apigenin, eupafolin, hispidulin, and luteolin) and the essential oil of the plant on different smooth muscles *in vitro*. The essential oil analyzed by our partners in Szeged, belongs to the chemotype characterized by the predominance of methallyl angelate.

The experiments were carried out with 60 μg/ml RKE on **guinea pig ilea**. Due to the incomplete reproducibility, RKE was given only once for every smooth

muscle preparation. RKE (60 µg/ml) caused contraction. The muscarinic receptor blocker atropine and the voltage-gated Na^+ channnel blocker TTX diminished the **excitatory responses** significantly. We applied PPADS to block the purinergic system, PPADS (50 µmol/l) did not hinder the contractions. The inhibition of serotonin receptors **[Sándor et al. 2016b]** resulted in no decrease in the contractions. The specific 5-HT_3 receptor blocker Y25130 (1 µmol/l), the specific 5-HT_4 receptor blocker SB204070 (1 µmol/l) and methysergide (0.3 µmol/l) administered together did not lessen the excitatory responses. We applied capsaicin desensibilisation for the functional blockade of capsaicin-sensitive afferent nerve endings **[see Barthó et al. 2004; Barthó et al. 2013]**. 60 µg/ml RKE was tested. Parallel with this test we used ethanol-solvent time control; the alcohols' concentration in the organ-bath was 0.5 µl/ml. Comparing the capsaicin-treated group and its control, there was no significant difference. The non-selective cyclooxigenase inhibitor indomethacin was used in 3 µmol/l concentration, parallel with its ethanolic control. The ethanol's concentration was in this case 0.3 µl/ml. In the presence of indomethacin the RKE-induced contraction significantly decreased (Mann-Whitney test).

The **relaxing** effect of RKE was measured in the presence of atropine and TTX (0.5-0.5 µmol/l both), after histamine (0.2 µmol/l) precontraction on **guinea pig ilea**. After approx. 20 min. incubation period, we obtained a plateau (50-70% of the maximal excitatory response); then 60 µg/ml concentration of RKE was administered. /We have to mention here that, in five cases, little spike-like transient contraction preceded the long-lasting relaxant response; but this transient excitatory effect did not exceed the 5% of the maximal contraction./ Propranolol and L-NOA had a 40 min. contact time. We did not find any significant difference among the propranolol-, L-NOA-treated group and the control group.

The **essential oil** of the plant in 10 µg/ml concentration diminished the amplitudes of the „twitch" contractions by 32.5%, on **guinea pig ilea**. The Wilcoxon-signed rank test indicated this reduction to be significant. Giving the essential oil alone in three different concentrations; 1 µg/ml, 10 µg/ml and 30 µg/ml did not cause any contraction on the ileum. We tested the relaxing effect of the essential oil on the histamine (0.2 µmol/l) precontracted ilea, in the presence of atropine and TTX and in the absence of them. We found the relaxations to be significant in the case of 1 µg/ml, 10 µg/ml essential oil concentration-groups, but the specimens treated with 0.1 µg/ml essential oil did not significantly relax in comparison with the control (DMSO) group.

The Roman chamomile's different **fractions** were examined in several concentrations estimating the contractile effect in the ileum. In the F60, F80, F100 fractions we experienced in higher concentrations declining concentration-response curve. The Roman chamomile's different fractions were also examined in several concentrations estimating the relaxant effect in the ileum. The inhibitory response of the fractions was evaluated in the presence of atropine and tetrodotoxin (0.5 μmol/l –both) and after histamine (0.2 μmol/l) precontraction. We also found here in higher concentrations from fractions F60, F80, F100 more expressed relaxant effect.

During assessing the contractile effect of Roman chamomile's **flavonoids**, we found that the flavonoids in the concentration of 1 μmol/l did not exert any contractile effect, but in 10 μmol/l concentration they evoked moderate excitatory response, 20-35 % of the maximal spasm of the ileum. The relaxant response of the flavonoids was evaluated in the presence of atropine and tetrodotoxin (0.5 μmol/l –both) and after histamine (0.2 μmol/l) precontraction.

In the guinea pig **urinary bladder** we examined the contractile effect of RKE in the concentrations of 20 μg/ml and 200 μg/ml. Capsaicin pretreatment approximately halved the contracions induced by RKE (200 μg/ml), in comparison with the control group.

For testing the relaxant effect of RKE on urinary bladder, we applied atropine and TTX (both 0.5 μmol/l), α,β-metATP desensitization, and histamine (1-3 μmol/l) precontraction. (The α,β-metATP desensitization blocks the purinergic excitatory responses in the bladder **[see Barthó et al. 2004, Merrill et al. 2016]**). After gaining a plateau, we administered 200 μg/ml RKE, whose relaxant response reached the two-thirds of the maximal relaxation; DMSO (1 μl/ml) control had no such inhibitory effect.

The essential oil also exerted only relaxant effect in the urinary bladder, it did not result any excitatory response in a concentration-range of 0.1-10 μg/ml. We achieved the sub-maximal contraction (approx. half of the maximal spasm) by giving histamine (10-20 μmol/l) and then after obtaining the plateau-phase, we administered the essenital oil. The transient spike-like contractions were absent after administering the different concentrations of the essential oil in the case of the urinary bladder, too. 10 μg/ml oil yielded twice bigger relaxation as the oil resulted in 0.1 μg/ml concentration.

We also evaluated the contractile effect of RKE on human jejunal longitudinal smooth muscle preparations. We examined the excitatory effect in the

concentrations of 20 μg/ml and 200 μg/ml. We administered the latter one twice after a 40 min. incubation period and multiple rinsing, but the response proved to be non-repeatable. The contractile effect of 200 μg/ml RKE was halved by TTX, which turned out to be statistically significant. Our previous examinations justified that, these contractile responses also lessened in the presence of atropine.

RKE's relaxant effect on human jejunal longitudinal smooth muscle preparations: After atropine and tetrodotoxin (0.5 μmol/l –both) treatment, the sub-maximal precontraction was evoked by $PGF_{2\alpha}$ (1-2 μmol/l for 20 min.). The 200 μg/ml RKE produced an average half-maximal relaxation. The DMSO control did not cause any alteration.

RKE's contractile and relaxant effect on human jejunal circular smooth muscle preparations was similar to the results recieved on longitudinal ones, but in this case 200 μg/ml RKE elicited maximal inhibitory response.

On human jejunal longitudinal smooth muscle preparations we assessed the relaxant effect of the essential oil. The oil – in the above mentioned three concentrations– did not cause any contraction. So, we tried to estimate the inhibitory effect of the oil, after $PGF_{2\alpha}$-evoked (1-2 μmol/l) precontractions, also in the presence of atropine and tetrodotoxin (0.5 μmol/l –both). We found more expressed relaxations in higher concentrations.

In the rat ileum, colon and acetylcholine-precontracted gastric fundus the essential oil caused only relaxations by definition; in the small bowel the 4 Hz "train" stimuli (for 30 s) were diminished also by 10 μg/ml essential oil, significantly. The DMSO controls did not cause any change.

Discussion

-I./a, It seems likely that AITC excites the human jejunum via TRPA1, as indicated by the inhibitory effect of the TRPA1 antagonists HC030031 or A967079. Capsaicin-sensitive nerves seem not to be involved; in fact, the effects of capsaicin and AITC are different from each other **[Maggi et al. 1990, Barthó et al. 2004].** (This holds true for the circular muscle as well **[Maggi et al. 1990, Barthó et al. 2004].**) Moreover, even axonal conduction through tetrodotoxin-sensitive Na^+ channels is not involved in the effect of AITC in longitudinally-oriented preparations. Purinoceptor antagonists that block the effect of AITC in the guinea pig ileum were also found ineffective in the human jejunum. The surprising inhibitory action of atropine and scopolamine and the enhancing effect of physostigmine led

us to the conclusion that AITC, via TRPA1, activates a cholinergic mechanism in the human small intestine, in a way that does not involve neuronal conduction. A possible explanation could be that cholinergic nerve endings harbour TRPA1 that stimulate acetylcholine release.

- I./b, According to Nozawa et al. (2009) the TRPA1 stimulant AITC exerts its contractile effect through the release of serotonin from enterochromaffin cells in the guinea pig ileum. In the present experiments, the nerve-mediated contraction of this preparation due to AITC was not inhibited by a combination of methysergide (broad-spectrum 5-HT antagonist; 0.3 μmol/l), Y25130 (azasetron, 5-HT_3 receptor antagonist; 1 μmol/l) and SB204070 (5-HT_4 receptor antagonist; 2 μmol/l) or by 5-HT receptor desensitization, that is, pretreatments that practically abolished contractions of similar size in response to exogenous 5-HT, without causing non-specific effects. AITC also contracted longitudinal muscle-myenteric plexus preparations, and the effect was also fully resistant to the combination of 5-HT receptor antagonists. The pharmacology of AITC in strip preparations matched that in the whole ileum. **(**Likewise, the excitatory action of the sensory stimulant capsaicin **[see Barthó et al. 2004]** was not influenced by the 5-HT antagonists, which also indicates that 5-HT does not play a part in the effect of this TRPV1 receptor/channel activator.) The concentrations of the 5-HT antagonists used in these experiments were well above the reported IC_{50} values **[see Prins et al. 1997, Alexander et al. 2018, Sato et al. 1992, Mylecharane 1989, Borman et al. 1995]**. 5-HT desensitization was also highly effective in blocking the action of exogenous (and probably also endogenous, if any) serotonin.

It has been stated **[Nozawa et al. 2009]** that the TRPA1 receptor/channel activator AITC contracts the guinea pig ileum by releasing 5-HT from enterochromaffin cells. This has been based on the strong inhibition of the contractile effect of AITC by ramosetron, a 5-HT_3 receptor antagonist. Our present data do not confirm either a major role of 5-HT in the excitation by AITC or an involvement of the gut mucosa therein. Neither a combination of 5-HT antagonists nor 5-HT desensitization reduced the effect of AITC in the whole ileum (but strongly reduced or abolished the effect of exogenous 5-HT). On the other hand, AITC contracted the LM-MP preparations **[see Donnerer, Liebmann 2017]** as well, and this effect was also uninfluenced by a combination of 5-HT antagonists or serotonin desensitisation (effective against exogenous 5-HT). In a previous study, we have reported that AITC caused nerve-mediated, largely cholinergic contraction in the guinea-pig ileum and that a purinergic mechanism was involved **[Barthó et al. 2013]**.

A similar inhibitory effect of the P_2 purinoceptor antagonist PPADS **[Alexander et al. 2018]** has now been found on LM-MP preparations. We conclude that neither 5-HT nor mucosal cells contribute to the excitatory effect of AITC in the guinea pig small intestine.

- I./c, Probably, in the guinea pig esophagus AITC exerts its contractile effect through neuronal elements and liberating tachykinins from nerve endings, as deduced from the effectiveness of voltage-gated sodium channel blocker tetrodotoxin and tachykinin receptor antagonists. AITC in this case has no direct smooth muscle effect in the concentrations tested. In this effect, neither cholinergic mechanism, nor TRPA1 receptors play a role (because of the ineffectiveness of atropine and specific TRPA1 antagonist).

- I./d, In the guinea pig urinary bladder prostanoids might be responsible for the AITC-evoked contractions, on the basis of the effectiveness of indomethacin. On the other hand, the TRPA1 antagonist A967079 –in the highest concentration applied– also hindered the AITC-evoked contracions, without non-specific effects. We could not examine further the overlap of this two mechanism due to lack of capacity.

- I./e, In the guinea pig trachea the AITC-evoked contractions were sensitive to capsaicin desensitization. It is likely, that AITC exerts its contractile action through the release of tachykinins (SP, neurokinin A). There is no contribution of P_2 purinergic receptors, voltage-gated Na^+ channels, M_3 muscarinic receptors in this process. TRPV1 or TRPA1 receptors are also not involved, as BCTC and HC030031 were without any effect. Due to the insignificant effect of indomethacin, prostanoids might not play a role in the excitatory effect of AITC in the guinea pig trachea.

- I./f, In the guinea pig gastric fundus the release of acetylcholine takes part in AITC's contractile action. Acetylcholine might be liberated from the myenteric plexus and makes the smooth muscle contract through muscarinic receptors. A purinergic mechanism is also involved in this contractile action. It is possible that, AITC excites PPADS-sensitive purinoceptors, which might be anchored to cholinergic nerve endings **[Sándor et al. 2016a]** and this leads to the release of acethylcholine.

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- I./g, In the precontracted guinea pig ileum AITC-evoked relaxation **[Donnerer, Liebmann 2017]** may occur through voltage-gated and Ca^{++} activated (TEA-

sensitive) K^+ channels. The TRPA1 receptor, P_2 purinoceptors, NO (owing to the ineffectiveness of their specific antagonists/synthesis inhibitor) do not contribute to the inhibitory action of AITC. Either TRPV1 receptor or capsaicin-sensitive nerve endings do not take part in this process. Adrenergic β_2 receptors are not involved due to the ineffectiveness of the β_1/β_2 receptor blocker propranolol.

- I./h, In the distal colon of the guinea pig AITC's inhibitory action is partly mediated by nerve conduction, as deduced from the efficacy of the voltage-gated Na^+ channel blocker: TTX. Altough, the inhibitory effect of apamin, indomethacin and capsaicin desensitization statistically turned out to be significant, this might not be a biologically meaningful phenomenon. (The shift in numeric data were rather mild). TRPA1 (as its specific antagonist did not abolish or lessen the relaxant response) or the purinergic system are also not implicated in this process in the colon.

- I./i, In the guinea pig taenia coeci the AITC-evoked relaxation is mediated through axonal conduction and voltage-gated and Ca^{++} activated K^+ channels.

(AITC caused no relaxant response in either untreated or precontracted urinary bladder, it resulted only contraction in the bladder.)

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- I./j, In the guinea pig ileum the contractile action of CINN is mediated through both purinergic and muscarinic receptors, axonal conduction, and TPRPA1, but the exact mechanism needs further experiments **[Sándor et al. 2019]**. The TRPA1-antagonist A967079 in 0.3 μmol/l concentration was free from non-specific effects in the ileum. The concentrations of this agent above 3 μmol/l already diminished the histamine-evoked contractions. The highest concentration of A967079 (10 μmol/l) possessed more non-specific effects. But, these results on specificity may be useful for other researchers in their work with TRPA1.

- I./k, In the guinea pig esophagus CINN resulted contractions independent from nerve conduction or cholinergic transmission. Capsaicin tachyphylaxis abolished the excitatory responses **[Sándor et al. 2019]**. We raise the possibility that CINN releases one or more tachykinins from the sensory nerve ending **[Barthó, Lénárd et al. 1999]**, which directly contracts the smooth muscle of the esophagus. We have not had yet the opportunity to investigate this issue in more details.

- I./l, In guinea pig urinary bladder the CINN-evoked contractions were sensitive for capsaicin desensitization **[Sándor et al. 2019]**. It is possible, CINN exerts its contractile action through the release of tachykinins, independently form TRPA1

and TRPV1 receptor activation, as their specific antagonists were ineffective. The wierd mechanism requires further clarification. Further, the excitatory response does not depend on the purinergic system, the nerve conduction and a cholinergic effect exerted directly on the smooth muscle cell itself.

- I./m, In the guinea pig trachea the CINN-evoked excitatory action was decreased by capsaicin desensitization **[Sándor et al. 2019]**. It is probable, that CINN –through stimulating the TRPA1 receptor– contracts the tracheal smooth muscle by releasing tachykinins from the capsaicin-sensitive nerve endings, but in part the muscarinic receptor contributes to the process. For example, the tachykinin may activate a cholinergic motoneuron on the axon terminal, which results in acetylcholine liberation contracting the smooth muscle. (The process does not depend on axonal conduction, P_2 receptors, TRPV1; due to the ineffectiveness of TTX, PPADS, and the TRPV1/TRPM8 blocker BCTC; respectively.)

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- I./n, In the precontracted guinea pig ileum CINN induced relaxations, **[Sándor et al. 2019]** which may occur through voltage-gated and Ca^{++} activated (TEA-sensitive) K^+ channels. The TRPA1 receptor, P_2 purinoceptors, NO (based upon the ineffectiveness of their specific antagonists) do not contribute to the inhibitory action of CINN. Either transmitters released from capsaicin-sensitive nerve endings **[see Barthó et al. 2004]** or adrenergic β_2 receptors do not take part in this process **[Sándor et al. 2019]**.

- I./o, In the guinea pig colon we could not prove the role of axonal voltage-gated Na^+ channels or P_2 purinergic receptors or small conductance Ca^{++} activated (apamin-sensitive) K^+ channels in the pharmacological background of CINN-evoked relaxations.

- I./p, In the precontracted urinary bladder of the guinea pig CINN administration yielded concentration-dependent relaxations. The inhibitory responses were not influenced by apamin, TTX or capsaicin desensitisation **[Sándor et al. 2019]**. So, we might assume neural voltage-gated Na^+ channels and small conductance K^+ channels or the relaxant effect of CGRP (calcitonin gene-related peptide) **[see Barthó et al. 2004]** are not involved in this action.

- I./q, In precontracted human jejunal samples –both in longitudinally- and circularly-oriented preparations– CINN induced relaxations. Our results indicate that the role of TRPA1 or TRPV1 receptors, voltage-gated Na^+ channels, voltage-

gated and Ca^{++}-activated K^+ channels in the background is improbable. (Further investigations shall be performed in future experiments.)

- I./r, CINN caused concentration-dependent relaxations in untreated guinea pig taenia coeci preparations.

-II. RKE has both stimulatory and relaxant effects in guinea pig ileum. The moderate, transient stimulatory activity results from the activation of cholinergic neurons, as confirmed by its inhibition by tetrodotoxin and atropine. Although capsaicin, a stimulant of a certain class of sensory receptors (through which it evokes a "local efferent" response) shows a similar cholinergic, neurogenic effect in guinea pig small intestine preparations **[see Barthó et al. 2004]**, a functional blockade of capsaicin-sensitive nerve endings by capsaicin pretreatment failed to inhibit the contractile action of RKE. The contractile action of RKE was moderately, yet significantly reduced by the cyclooxygenase inhibitor indomethacin, which may indicate a modulatory role of endogenous prostanoids. Other pharmacological inhibitors tested had no contraction-reducing effect, therefore it is proposed that neither endogenous serotonin, nor PPADS-sensitive purinergic mechanisms play a role in the excitatory action of RKE. It should be noted that both serotonin and ATP or the P_{2X} receptor agonist α,β-metATP are able to evoke cholinergic contractions in guinea pig ileum **[see Benkó et al. 2005; Barthó et al. 2006; Sándor et al. 2016b].**

Following the transient, mild-to-moderate stimulant effect, the RKE exhibited a sustained relaxant effect on the guinea pig ileum in the presence of tetrodotoxin and atropine, and preliminary experiments indicated a similar relaxant effect also in the absence of atropine and tetrodotoxin. Yet, atropine and tetrodotoxin were included into these experiments for creating a methodologically clear situation, where an initial, neuronally mediated excitatory action would hardly interfere with the relaxant one. The concentrations of the extract causing relaxation were roughly the same as those causing contraction. Based on these data we propose that the site of action for the relaxant effect of RKE is on the smooth muscle itself. Neither the adrenergic β-receptor antagonist propranolol nor the NO synthase inhibitor N^G-nitro-L-arginine significantly reduced the relaxant effect of RKE, hence, no evidence was found for these mechanisms to be involved in the relaxant response. In fact, a direct relaxant effect of RKE was demonstrated on all gastrointestinal preparations tested, including human and rat gastrointestinal preparations, as well as in guinea pig urinary bladder. In preparations with high

intrinsic tone (rat ileum and rat colon), relaxant activity was practically the only response to be seen.

Different RKE fractions evoked both contraction and relaxation in guinea pig ileum. While there was a clear-cut tendency correlation between the flavonoid content and the relaxant effect, in case of contractile action such correlation was not observed (on the contrary, fractions with lower flavonoid content exerted slightly higher contractile activities). Fractions with high flavonoid content (F60, F80, and F100) had remarkable relaxant effects, whereas fractions with no (F20) or low (F40) flavonoid content exerted no such activity.

The pure flavonoids of the extract showed dual effects in the guinea pig ileum, much similarly to RKE. Moreover, there was no substantial difference between the potencies of the flavonoids. This means that any of these flavonoids may contribute to the sustained relaxant and the transient contractile effect of RKE.

The essential oil of Roman chamomile caused no contraction on the test preparations; nevertheless, a consistent relaxant effect was detected. This seems to indicate that chemical components responsible for the stimulant effect may be absent in the essential oil.

The contraction of guinea pig bladder in response to the RKE proved to be special, in that it was reduced by half following *in vitro* capsaicin pretreatment. This indicates that capsaicin-sensitive sensory nerves of the bladder wall are in some way involved in the excitatory effect of RKE **[see Barthó et al. 2004].** Nevertheless, RKE and the volatile oil also induced bladder relaxation in precontracted preparations in the same concetration as they exerted their contractile effect.

RKE was effective in human jejunal preparations as well. Both an excitatory and a tetrodotoxin and atropine-resistant relaxing effect were demonstrated; again, the smooth muscle relaxant one being more sustained than the excitatory one. Preliminary experiments have shown that this early contraction is reduced by atropine. Due to limited time and access to human tissue, no further analysis of these responses could be performed.

Summary of new findings, possible importance in clinical practice

Our new findings were as follows:

In human jejunum, probably there are TRPA1 receptors on cholinergic nerve endings, these receptors are releasing acetylcholine **[Sándor et al. 2016a].**

In the guinea pig ileum, axonal conduction, cholinergic and purinergic mechanisms contribute to the AITC-evoked contractile activity. Neither endogenous serotonin, nor gut mucosa take part in the excitatory effect of AITC **[Sándor et al. 2016b].**

Tachykinins play a role in the contactile action of CINN in the case of guinea pig esophagus, trachea and urinary bladder **[Sándor et al. 2019].** The AITC-evoked contraction is mediated by tachykinins in the case of trachea and esophagus.

Purinergic mechanism takes part in the contractile effect of TRPA1 agonists in the case of guinea pig ileum **[Sándor et al. 2016b, Sándor et al. 2019]** and in the excitatory action of AITC in the gastric fundus.

Axonal conduction is involved in the contractile action of the TRPA1 agonists in the guinea pig ileum **[Sándor et al. 2016b, Sándor et al. 2019],** and in the excitatory action of AITC in the esophagus. The voltage-gated Na^+ channels also contribute to the contractile effect of RKE in human and guinea pig small intestine **[Sándor, Mottaghipisheh et al. 2018].**

TRPA1 receptor mediation is likely in the excitatory action of AITC regarding the guinea pig urinary bladder, and in the view of the contractile effect of CINN in the ileum **[Sándor et al. 2019]** and probably in the trachea. (Moreover, the exciting effect of AITC is generated through this receptor in human small intestine –as mentioned above.)

The cholinergic process is involved in the background of TRPA1 agonists' contractile effect in guinea pig ileum **[Sándor et al. 2016b, Sándor et al. 2019].** Acetylcholine also contracts the gastric fundus after AITC administration. Applying CINN results cholinergic contraction of guinea pig trachea. Acetylcholine is also responsible for the excitatory effect of RKE in the human and guinea pig small bowels **[Sándor, Mottaghipisheh et al. 2018].** (Further, the human

small intestine is contracted by cholinergic mechanism in the contractile effect of AITC.)

Prostanoid production may play a role in the contractile effect of AITC in the guinea pig urinary bladder and in the excitatory action of RKE regarding guinea pig ileum **[Sándor, Mottaghipisheh et al. 2018].**

Based on the effectiveness of capsaicin tachyphylaxis, we found evidence for the involvement of capsaicin-sensitive nerve endings in the contractile effect of RKE in the case of the guinea pig urinary bladder **[Sándor, Mottaghipisheh et al. 2018],** in the excitatory effect of CINN regarding the bladder, trachea and esophagus **[Sándor et al. 2019].** Finally, these structures also play a role in the contractile effect of AITC in the guinea pig trachea.

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In the relaxant response of TRPA1 agonists, TEA-sensitive K^+ channels play a role in the case of guinea pig ileum **[Sándor et al. 2019]** and –regardig the inhibitory response of AITC– in the case of taenia coeci.

AITC-evoked relaxation is implemented by axonal conduction in the guinea pig colon and taenia coeci.

The results summarized in this thesis are the parts of basic research, they might contibute to the better understandig of gastrointestinal movements. In the future, they may support the exploration, diagnosis and therapy of pathologically altered conditions.

In terms of possible medical utilization of Roman chamomile it may be noted that a combination of nerve-mediated, mild-to moderate excitatory effect and a smooth muscle-relaxant action may even be advantageous for some gastrointestinal problems, e.g., diminished peristaltic activity, while for spasms in the stomach or large intestine it is obviously the smooth muscle-relaxing activity that offers benefits. The medicinal plant exerts its relaxant effect on the smooth muscle itself. Our results support the overall smooth muscle-relaxant effect of RKE and of the essential oil of the herb. The predominant effect of RKE, its fractions and flavonoids is relaxation, being more sustained than the transient contraction

observed in some cases. This activity is in correlation with the flavonoid content of RKE. Since the components of the essential oil are partly extracted with alcohols, the constituents of the oil also contribute to the overall effect of RKE. The extract used in our experiments was prepared in accordance with the European Medicines Agency monograph for this plant, therefore our results support the traditional usage of this plant **[Sándor, Mottaghipisheh et al. 2018].**

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