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DOTTORATO DI RICERCA IN FISIOPATOLOGIA NEUROSENSORIALE

ANALISI DEI MECCANISMI DI CONTROLLO DELLA MOTILITA' GASTROINTESTINALE DA PARTE DI ORMONI COINVOLTI NEL BILANCIO IDRICO-SALINO

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SUMMARY

The enteric nervous system (ENS) is an integrative neuronal network, composed of neurons and enteric glial cells, controlling the activity of the smooth muscle of the gut, mucosal secretion and blood flow. This highly integrated neural system is also referred to as the ‘brain-in-the-gut’, because of its capability to function in the absence of nerve inputs from the central nervous system. Hormones and paracrine mediators released by different source cells may directly influences central neuronal function and thereby gut motility. However for many of them the presence of specific receptors at different levels in the ENS, smooth muscle cells or other effectors (i.e. ICC) has been demonstrated, indicating a peripheral site of action. The small and large intestine play a key role in maintenance of body fluid homeostasis by regulating the transport of ions by the intestinal epithelium. It is known that absorption of nutrients, water and electrolytes in the intestine can be affected by alteration of motility. Among the hormones involved in the control of body fluid and electrolyte homoeostasis, angiotensin II (Ang II) and vasopressin (AVP) have been demonstrated to be able to modulate the processes of absorption/secretion of ions and water in the intestine, thus, it can be suggested their involvement in the modulation of the intestinal contractility as part of their regulatory mechanism. However, several aspects pertaining to the regulation of digestive functions by Ang II and AVP remain unclear and deserve extensive investigation. Considering that the presence of receptors for Ang II and AVP at various levels along the GI tract in different animal species, including human, we addressed the possibility that Ang II and AVP might be involved in the regulation of intestinal (colon) motility acting at peripheral level, using the organ-bath technique to measure changes in isometric tension of longitudinal and circular colonic muscle. We choose such an approach in vitro since the influence of external

factors was removed, but the intestinal muscle itself performed in a manner analogous to its in vivo capacity. Both mouse and human colonic muscle segments/strips have been used and, in detail, we evaluated the effects of Ang II and AVP on the contractility of the large intestine and we analysed the subtype(s) of receptor(s) activated and the action mechanism. Moreover, by RT-PCR, the expression of transcripts of the main components of the rennin-angiotensin system (RAS) has been analysed in the mouse and human colon. Results from our studies showed that in the murine proximal and distal colon Ang II induced a concentration-dependent muscular contraction, reduced by the AT1 receptor antagonist, losartan, but not affected by the AT2 receptor antagonist, PD123319. Pre-treatment with TTX, sodium voltage-gated neural channel blocker, partially reduced the contractile response to Ang II in the proximal colon, while abolished it in the distal colon. Atropine, muscarinic receptor antagonist, or SR140333, NK1 receptor antagonist, reduced the TTX-sensitive excitatory effects induced by Ang II in both preparations. On the contrary, hexamethonium, nicotinic receptor antagonists, ondansetron, 5-HT3 receptor antagonist, or SR48968, NK2 receptor antagonist, were ineffective. The contraction induced by a selective NK1 receptor agonist was reduced by atropine, whilst SR140333 did not affected carbachol induced muscular contraction. Transcripts encoding RAS components were detected in the colon samples. However, just AT1A mRNA was expressed in both preparations, and AT2 mRNA was expressed only in the distal colon. In the human sigma colonic circular muscle preparations, Ang II, as well, induced a muscular contraction, antagonized by losartan but not by PD123319. TTX partially reduced the contractile response. SR48968, NK2 receptor antagonist, was able to reduce significantly the excitatory effects induced by Ang II, being atropine ineffective. Lastly, the contraction to the specific NK2 receptor agonist, β-Ala, was not affected by TTX or atropine. Transcripts encoding

for AT1 and AT2 Ang II receptor subtypes and for angiotensin-converting enzyme (ACE) were expressed in the whole thickness preparations. No expression of mRNA for angiotensinogen (AGT) and for rennin was found. AVP caused concentration-dependent contractile effects only on the longitudinal muscle of murine colon, being ineffective at the circular one. AVP-induced contraction was antagonized by the V1 receptor antagonist, V-1880. AVP-induced effect was not modified by tetrodotoxin, atropine and indomethacin. Contractile response to AVP was reduced in Ca^{2+}-free solution or in the presence of nifedipine, L-type calcium channel antagonist, and it was abolished by depletion of calcium intracellular stores after repetitive addition of carbachol in calcium-free medium with addition of cyclopiazonic acid.U-73122, an inhibitor of the phospholipase C, effectively antagonized AVP effects, whilst DDA, an adenylyl cyclase inhibitor, was ineffective. Oxytocin induced an excitatory effect in the longitudinal muscle of mouse distal colon at very high concentrations, antagonized by V-1880. In conclusion**,** Ang II positively modulates the spontaneous contractile activity of mouse and human colon via activation of post-junctional and pre-junctional AT1 receptors (AT1A receptors in mice), the latter located on the enteric nerves modulating the release of excitatory neurotransmitters. In details, in mouse tachykinergic neurons and cholinergic neurons are sequentially recruited by Ang II to induce muscular contraction. Ang II would induce release of substance P by enteric nerves, which acting on NK1 receptors, in turn would induce release of acetylcholine, being the final contractile mediator. In human sigmoid colon Ang II positively modulates the spontaneous contractile activity via activation of post-junctional and pre-junctional AT1 receptors, the latter located on the enteric nerves and modulating the release of tachykinins, which in turn, via activation of NK2 receptors, would contribute to the contractile effects. PCR analysis showed that transcripts encoding for AT1 and AT2 receptor subtypes and for

components of RAS were expressed both preparations, suggesting a likely local source of Ang II. Moreover, AVP, via activation of V1 receptors, it is able to modulate positively contractile activity of longitudinal muscle of mouse distal colon, independently by enteric nerve activation and prostaglandin synthesis. Contractile response is achieved by increase in cytoplasmatic Ca^{2+} concentration via extracellular Ca^{2+} influx from L-type Ca^{2+} channels and via Ca^{2+} release from intracellular stores through phospholipase C pathway. No modulation has been observed on the contractility of the circular muscle. In conclusion, results present in this thesis indicate that the modulation of the colonic contractility and the consequent impact on intestinal transit can be one of the physiological mechanisms by which Ang II and AVP would control body fluid and electrolyte homoeostasis.

SOMMARIO

Il sistema nervoso enterico (ENS) è una complessa rete neuronale, composta di neuroni e di cellule gliali enteriche, che controllano l'attività della muscolatura liscia intestinale, la secrezione della mucosa ed il flusso sanguigno. Questo sistema neurale altamente integrato è denominato anche "il cervello nell' intestino" o il "piccolo cervello", a causa della sua capacità di funzionare indipendentemente dal sistema nervoso centrale. Ormoni e mediatori paracrini rilasciati da cellule diverse, possono influenzare direttamente la funzione nervosa a livello centrale e quindi la motilità intestinale. Tuttavia, per molte di queste sostanze è stata dimostrata la presenza di recettori specifici a diversi livelli nella parete intestinale, ENS, cellule muscolari lisce o altri effettori (ICC), indicando un'azione periferica. L' intestino tenue e crasso giocano un ruolo chiave nel mantenimento dell'omeostasi idrico-salina, regolando il trasporto di ioni a livello dell' epitelio intestinale. E noto come l'assorbimento di nutrienti, di acqua ed elettroliti nell'intestino possa essere influenzato da alterazione della motilità. Tra gli ormoni coinvolti nel controllo dell'omeostasi di liquidi ed elettroliti, è stato dimostrato che l'angiotensina II (Ang II) e la vasopressina (AVP) sono in grado di modulare i processi di assorbimento/secrezione di ioni e acqua nell'intestino, quindi, è possibile ipotizzare un loro coinvolgimento nella modulazione della contrattilità intestinale come parte del meccanismo di regolazione omeostatica. Tuttavia, ancora rimangono poco chiari e, pertanto, meritano una approfondita indagine, i diversi aspetti che riguardano nel suo complesso la regolazione delle funzioni intestinali da parte dell'Ang II e della AVP. Considerando la presenza, in diverse specie animali compreso l'uomo, dei recettori per l'Ang II e per la AVP lungo il tratto GI, abbiamo valutato la possibilità che l'Ang II e la AVP possano essere coinvolti nella regolazione della motilità intestinale (colon), agendo a

livello periferico, mediante l’utilizzo della tecnica del bagno per organo che consente la misurazione delle variazioni di tensione isometrica del muscolo longitudinale e circolare intestinale. Abbiamo scelto tale approccio in vitro, poiché in tali condizioni è rimossa l'influenza di fattori esterni, ma l’ intestino riesce a funzionare in maniera analoga alla sua capacità in vivo. Segmenti e/o strips di muscolatura di topo e di uomo sono stati utilizzati per valutare gli effetti dell’Ang II e della AVP sulla contrattilità del colon, analizzare il sottotipo (i) recettoriale (i) coinvolti e il meccanismo di azione ad esso correlato. Inoltre, mediante l’utilizzo di RT- PCR abbiamo valutato l'espressione dei trascritti dei principali componenti del sistema renina - angiotensina (RAS). I risultati dei nostri studi hanno dimostrato che in porzioni di colon prossimale e distale di topo l’Ang II induceva una contrazione muscolare concentrazione-dipendente, che risultava essere ridotta in presenza dell'antagonista del recettore AT1, losartan , ma non in presenza dell’antagonista del recettore AT2 , PD123319. Il pretrattamento con TTX, bloccante dei canali ionici neurali per il Na^+ voltaggio-dipendenti, riduceva parzialmente la risposta contrattile dell’Ang II nel colon prossimale, mentre la aboliva a livello del colon distale. In entrambe le preparazioni, in presenza di atropina, antagonista dei recettori muscarinici, o di SR140333, antagonista dei recettori tachichinergici NK1, gli effetti eccitatori indotti dall’Ang II risultavano ridotti. Al contrario nessun effetto era osservato in presenza di esametonio, antagonista dei recettori nicotinici, dell’ondansetron, antagonista dei recettori 5-HT_3 o dell’ SR48968 , antagonista dei recettori tachichinergici NK2 . La contrazione indotta da un agonista selettivo del recettore NK1 era ridotta in presenza di atropina, mentre l’SR140333 non influenzava la contrazione muscolare indotta dall’agonista colinergico carbacolo. Nei campioni di colon sono stati rilevati i trascritti per tutti i componenti del sistema RAS . Tuttavia l’mRNA del recettore AT1A risultava essere espresso in entrambi preparati, mentre l’m

RNA del recettore AT2 era espresso solo a livello del colon distale. Nei preparati di muscolatura circolare di sigma colon di uomo, l'Ang II, induceva una contrazione muscolare, antagonizzata dal losartan ma non dal PD123319. In presenza di TTX l'effetto contrattile dell'Ang II risultava essere parzialmente ridotto. SR48968, antagonista del recettore NK2, è stato in grado di ridurre significativamente gli effetti eccitatori indotti dall' Ang II, mentre l'atropina risultava inefficace. Infine, la contrazione indotta dall'agonista β-Ala, agonista specifico del recettore NK2, non risultava antagonizzata in presenza di TTX o di atropina. I trascritti codificanti per i sottotipi recettoriali AT1 e AT2 dell'Ang II e per l'enzima ACE sono risultati espressi nei nostri preparati. Non è stata trovata alcuna espressione per gli mRNA dell' angiotensinogeno (AGT) e della renina .
L'AVP causava effetti contrattili concentrazione-dipendente solo a livello della muscolatura longitudinale del colon di topo, risultando inefficace a livello della muscolatura circolare. Gli effetti erano antagonizzati dal antagonista del recettore V1, V -1880. Gli effetti indotti dalla AVP non risultavano essere modificati in presenza di TTX, atropina o di indometacina. In una soluzione priva di Ca^{2+} o in presenza di nifedipina, antagonista dei canali al calcio di tipo L, la risposta contrattile della AVP è stata ridotta, mentre veniva abolita in seguito alla deplezione di calcio dai depositi intracellulari, ottenuta mediante addizione ripetitiva di carbacolo in un terreno privo di calcio e con l'aggiunta di CPA. U - 73122, un inibitore della fosfolipasi C, antagonizzava gli effetti indotti della AVP, mentre DDA, un inibitore dell'adenilato ciclasi, era inefficace. L'ossitocina induceva un effetto eccitatorio a livello del muscolo longitudinale del colon distale di topo soltanto a concentrazioni molto elevate, effetto antagonizzato dal V-1880. In conclusione, l'Ang II modula positivamente l'attività contrattile spontanea del colon di topo e umano attraverso l'attivazione dei recettori AT1 (recettori AT1A nei topi) localizzati a livello post- e pre- giunzionale,

quest'ultimo localizzato a livello dei neuroni enterici modulando il rilascio di neurotrasmettitori eccitatori. Nel dettaglio, nel topo neuroni tachichinergici e neuroni colinergici sono sequenzialmente reclutati da parte dell'Ang II per indurre la contrazione muscolare. Ang II indurrebbe il rilascio di sostanza P dai neuroni enterici, che agendo sui recettori NK1, indurrebbe a sua volte il rilascio di acetilcolina, il mediatore finale coinvolto nella genesi della risposta contrattile. A livello del sigma colon di uomo, l'Ang II modula positivamente l'attività contrattile spontanea mediante l'attivazione di recettori AT1 localizzati a livello post-giunzionali e pre-giunzionali, questi ultimi situati sui neuroni enterici, modulano il rilascio di tachichinine, che a loro volta, attraverso l'attivazione di recettori NK2, contribuirebbero agli effetti contrattili. Analisi di PCR hanno evidenziato la presenza dei trascritti codificanti per i sottotipi recettoriali AT1 e AT2 e per i componenti del sistema RAS in entrambe le preparazioni, suggerendo una probabile produzione locale di Ang II. L'AVP, attraverso l'attivazione di recettori V1, è in grado di modulare positivamente l'attività contrattile del muscolo longitudinale di colon distale di topo, indipendentemente dall'attivazione di neuroni enterici e dalla sintesi di prostaglandine. La risposta contrattile si ha in seguito all'aumento della concentrazione di Ca^{2+} citoplasmatico, attraverso l'afflusso di Ca^{2+} extracellulare dai canali di tipo L Ca^{2+} e mediante rilascio di Ca^{2+} dai depositi intracellulari attraverso il pathway della fosfolipasi C. Nessuna modulazione è stata osservata sulla contrattilità del muscolo circolare. In conclusione, i risultati presentati in questa tesi indicano che la modulazione della contrattilità del colon e il conseguente impatto sul transito intestinale può essere uno dei meccanismi fisiologici con cui Ang II e AVP regolerebbero l'omeostasi idrico-salina.

Capitolo 1

Introduction

An important function of the gut is to transport ingested food at an optimal rate and to mix the food for optimal exposure to digestive enzymes. This is achieved by the contractile activity of smooth muscle cells of the gut wall. The regulation of smooth muscle activity and, thus, of gut motility takes place at several levels. The complex neural network, known as enteric nervous system (ENS), endowed in the gut wall and extending throughout its length from the oesophagus to the internal anal sphincter is the dominating component which acts directly and indirectly on muscle cells. In addition, local and circulating neurohumoral substances play a part in the complex regulation of gastrointestinal motility, also interacting with enteric neurotransmitter to modify excitatory or inhibitory signals to the muscle cells. In view of this complexity it is not surprising that any impairment of the modulation of ENS circuitries results in a wide array of gut disorders, including motor impairments, which are characterized by high morbidity, with a markedly compromised patient's quality of life and occasional fatal outcomes. So far, our knowledge about the mechanisms of actions of the various neurohormones and drugs affecting gut motility is still fragmented and incomplete. However, recently substantial progress has been achieved, and drug therapy for gut dysmotility is emerging, based primarily on managing neurohumoral receptors. The purpose of this thesis is to present my studies on the mechanism by which GI motility is controlled and modulated by neurohumoral substances with focus on the large intestine and hormones involved in the regulation of body fluid and electrolyte balance and circulation, such as Angiotensin II (Ang II) and Vasopressin (AVP), in consideration that gastrointestinal tract is fundamental for intake and

excretion of fluid and electrolytes and it accommodates a large proportion of bodily haemodynamic system.

The Enteric Nervous System and the Control of Gut Motility

The enteric nervous system is a network of neurons and glia within the wall of the bowel that is able to control most aspects of intestinal function. In humans, the ENS contains 500 million neurons characterized by a wide range of neurotransmitters, projection patterns, and electrical properties. Approximately 20 types of enteric neurons can be defined by their functions (Brookes and Costa 2002; Furness 2006). Combinations of features (morphology, neurochemical properties, cell physiology and projections to targets) help to define each type of neurons. Enteric neurons can be classified as intrinsic primary afferent neurons (IPANs), which monitor the state of the lumen and the gut wall, ascending and descending interneurons, which connect enteric neurons within the different ganglia, and excitatory and inhibitory motor neurons, which target the effectors. IPANs detect the physical state of the organs (for example, tension in the gut wall) and chemical features of the luminal contents (Furness et al. 2004). They react to these signals to initiate appropriate reflex control of motility, secretion and blood flow. IPANs connect with each other, with interneurons and directly with motor neurons. Interneurons connect with other interneurons and with motor neurons. Among the motor neurons are muscle motor neurons, secretomotor neurons, secretomotor/vasodilator neurons and vasodilator neurons. ENS is unique in its ability to function independently of the central nervous system (CNS) in the control of the functions of the digestive tract. For this reason, the ENS is considered to be a “second brain in the gut”.

However, the CNS is able to modulate, but not entirely control, the GI function by sending instructions via the two components of the extrinsic

autonomic nervous system: the sympathetic and parasympathetic nervous system.

The ENS has multiple roles:

- determining the patterns of movement of gastrointestinal tract;
- controlling gastric acid secretion;
- regulating movement of fluid across the lining epithelium;
- changing local blood flow;
- modifying nutrient handling;
- interacting with the immune and endocrine system of the gut.

The enteric nervous system of the digestive tract (the esophagus, stomach and intestines) influences effector systems in the gut directly or indirectly through its action on intermediate cells, which include neuroendocrine cells, interstitial cells of Cajal (ICC), and cells of the immune system. ENS expresses all neurotransmitters so far known in CNS (more than 30 neurotransmitters). These include classical neurotransmitters such as acetylcholine (Ach), noradrenaline, serotonin, GABA and glutamate, but a great number of other neurotransmitter and hormones also participate in the regulation of functions in the GI tract: vasoactive intestinal polypeptide (VIP), nitric oxide, galanin, motilin, adenosine triphosphate, tachykinins, ecc. The ENS is organized in two ganglionated plexuses, myenteric and submucosal, composed of neurons and enteric glial cells. The myenteric plexus (or Auerbach's plexus is positioned between the outer longitudinal and circular muscle layers throughout the digestive tract, from the oesophagus to the rectum. The submucosal plexus (or Meissner's plexus) is positioned in the submucosa, being only prominent in the intestines. Neurons of the myenteric plexus control the activity of the smooth muscle of

the gut, whereas those in the submucosal plexus regulate mucosal secretion and blood flow (Fig.1).

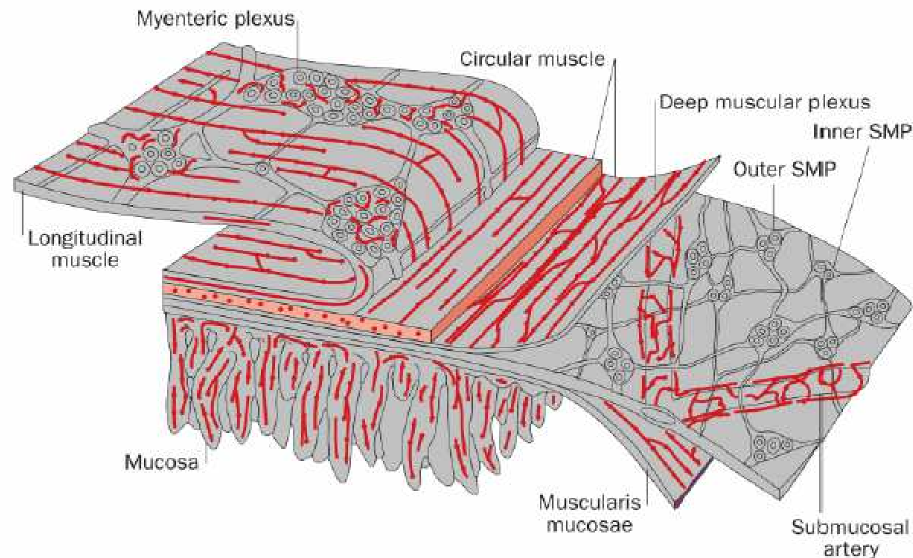


Fig.1- Detailed organization of nerve cell plexuses in the gut wall"

The ENS controls gut motility and secretion via local reflexes that are triggered by local distension of the intestinal wall, distortion of the mucosa, and chemical contents in the lumen. These reflexes involve parallel circuits of synaptically interconnected ENS neurons, which include primary intrinsic afferent neural cells, ascending and descending interneurons, excitatory and inhibitory motorneurons, vasomotorneurons and secretomotorneurons.

For example, in the myenteric plexus, activation of ascending interneurons and excitatory motoneurons results in the release of excitatory neuromediators (acetylcholine, substance P) on smooth muscle fibers causing circumferential contraction of the circular muscle layer upstream of the bolus. Activation of descending interneurons and inhibitory motoneurons results in the release of inhibitory neuromediators (VIP - vasoactive intestinal polypeptide, nitric oxide) causing relaxation of circular muscle downstream of the bolus.

The enteric nervous system receives inputs from the parasympathetic and sympathetic parts of the nervous system, and the gastrointestinal tract also receives a plentiful supply of afferent nerve fibres, through the vagus nerves and spinal afferent pathways. The parasympathetic innervation via the vagus nerves influences the motor and secretomotor function of the upper GI tract. The sympathetic adrenergic fibres from the prevertebral ganglia innervate the secretomotor neurons containing vasoactive intestinal polypeptide, presynaptic cholinergic nerve endings, submucosal blood vessels, and the sphincters of the GI tract. Thus, there is a rich interaction, in both directions, between the enteric nervous system, sympathetic prevertebral ganglia and the CNS. Lastly, enteric neurons also interact with the extensive intrinsic immune system of the gastrointestinal tract. The evidence suggests that immunoneural signaling triggers a neural program for defensive intestinal behavior in response to circumstances within the lumen that are threatening to the functional integrity of the whole animal (Wood J, 2004).

The ENS in the intestinal water and electrolyte balance

Called “the misunderstood nephron” by Michell (2000), daily fluid flux across the gastrointestinal tract making it ideally placed to regulate water and electrolyte balance, a cooperative task shared with the kidneys. The regulation of intestinal salt and water transport is critical for the maintenance of fluid volume. The interaction between absorption and secretion is controlled by classical neurotransmitters, predominantly released by the enteric submucosal motor neurons, hormones and paracrine substances, which in general act on membrane-bound receptors in order to modify intracellular second messenger systems regulating ionic transport across the epithelium. Nevertheless, interconnections between submucosal and myenteric plexuses provide the networks for coordination of motility

and ion transport function. Moreover, the ENS, regulating GI motility (Hansen 2003a), blood flow (Hansen et al. 1998), secretions (Hansen & Skadhauge 1995; Cooke 2000), and maintenance of body fluid homeostasis via transport of ions trough the intestinal epithelium, can be modulate by different neurotransmitters, paracrine substances and hormones. Among the hormones, some substances, known to have a role in the control of salt-water balance, acting mainly on kidneys, can contribute to the adsorptive/secretive processes activating receptors in the GI tract.

The control of fluid and electrolyte balance is closely connected with gastrointestinal function which acts in concert with the kidneys in enterorenal axis. The Renin- Angiotensin- Aldosterone System plays the most important role in maintaining the water-salt balance and regulation of blood pressure, also stimulating the intestinal colon absorption of sodium and water (D Dolman & C J Edmonds 1975; Levitan & Ingelfinger , 1965; Davies et. al, 1970; Hornych et. al, 1973). Others hormones, such as vasopressin (Vp), which is a critical regulator of water homeostasis by controlling the insertion of aquaporin2 (AQP2) onto the apical membrane of the renal collecting duct (Nielsen et al. 1993, 1995, Fushimi et al.1997), has been reported to regulate electrolyte and water transport in the colon both in vivo and in vitro (Dennhardt R et. al, 1979; Levitan R et. al, 1968; Bridges RJ et. al, 1983 and 1984; Knobloch SF et. al, 1989; Mobasheri A et. al, 2005; Vincentini-Paulino 1992). However, few studies have been addressed on the possibility that the modulation of the intestinal contractility and the consequent impact on motility pattern can be one of the mechanisms by which hormones, as Ang II or AVP, would control body fluid and electrolyte homeostasis.

Angiotensin II

Angiotensin II (Ang II), the major bioactive component of the renin–angiotensin system (RAS), induces a multitude of events contributing to the regulation of blood pressure, body fluid volume and electrolyte balance. Traditionally, RAS is regarded as an endocrine system, but its components can be found in several tissues, indicating also paracrine–autocrine functions (Paul et al. 2006, Fyhrquist & Saijonmaa 2008). In addition to the systemic (circulating) RAS, there is evidence to indicate that many tissues, including the vasculature, heart, kidney and brain, are capable of producing Ang II, which may thereby mediate autocrine, paracrine and intracrine effects (Campbell, D. J. 1987; Johnston, C. I. 1992). RAS as endocrine system *via* its key mediator Ang II targets primarily the renocardiovascular system to maintain fluid and electrolyte homeostasis (Fig.3). Stress on body fluids, particularly a reduced blood volume, as manifested by a lowered arterial pressure or a sodium deficiency, will initiate the release of the proteolytic enzyme renin from the juxtaglomerular apparatus of the kidneys. Renin cleaves off the decapeptide angiotensin I (Ang I) from the precursor protein angiotensinogen released by the liver. Ang I is then degraded to the octapeptide Ang II by angiotensin-converting enzyme (ACE) expressed by endothelial cells mainly in pulmonary vessels.

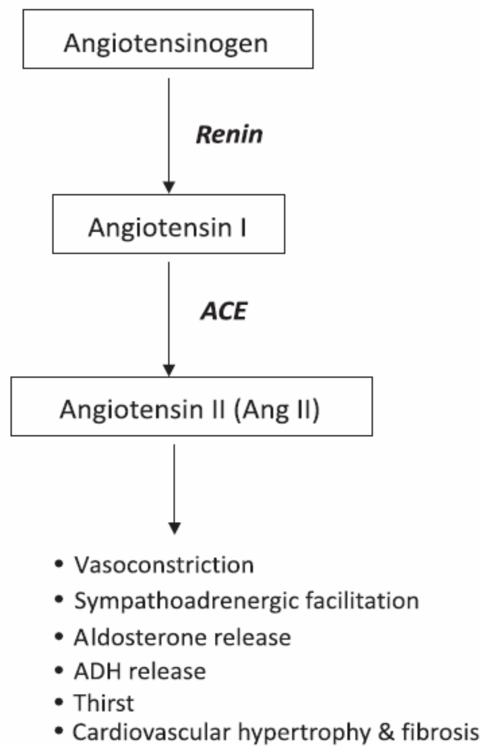


Fig.3- The “classical” endocrine renin-angiotensin system

Once released, circulating Ang II contributes to the maintenance of an adequate arterial pressure by selective constriction of certain vascular beds, preferentially in the splanchnic organs. During circulatory stress, this mechanism is considered to be of ultimate importance for the individual’s short-term survival by protecting the cardio-pulmonary-cerebral circulation and thereby oxygen delivery to the central nervous system (CNS). In addition, Ang II induces renal retention of sodium and fluid to compensate for the reduced blood volume by via binding to AT1 receptors. Activation of AT1 receptors leads to increased activities of the proximal tubule apical sodium/hydrogen exchanger and of the basolateral sodium bicarbonate cotransporter. Ang II also enhances the activity of the sodium-chloride co-transporter in the distal tubule and the epithelial sodium channel in the collecting duct (Lisa M. Harrison-Bernard 2009).

Ang II also mediates the thirst sensation and salt appetite driving the individual to a final fluid compensation by increased oral intake of water and sodium. Ang II exerts its impact both directly on the renocardiovascular system as well as indirectly via other regulatory factors, for example by facilitation of sympathetic nervous activity or by liberation of aldosterone from the adrenals. Because of the latter pathway, the system is also termed 'the renin–angiotensin–aldosterone system' (RAAS) (Weber 2001).

Angiotensin II receptors

Two main Ang II receptors have been described, namely AT1 and AT2 receptors, both members of the G protein-coupled receptor family (De Gasparo et al. 2000). Ang II receptors are members of the seven transmembrane G protein– coupled receptors (GPCR). There is relatively low sequence homology between the AT1 and AT2 receptors (Mukoyama M,. 1993). The differential expression of type of angiotensin-generating enzymes, and/or type of receptors, will determine the functionality of RAS in a given tissue (Song et. al, 1992; Aldred G et. al, 1993, Zhuo et. al., 1992, 1993 and 1997). Virtually all the known biological actions of Ang II, including vasoconstriction, release of aldosterone, stimulation of sympathetic transmission and cellular growth, are exclusively mediated by the AT1 receptor. The functional role of the AT2 receptor is not fully understood, but recent studies have ascribed a possible role of AT2 receptors in mediating anti-proliferation, apoptosis, differentiation and possibly vasodilatation (Horiuchi, M. 1996; Csikos, T 1998) (Fig.4).

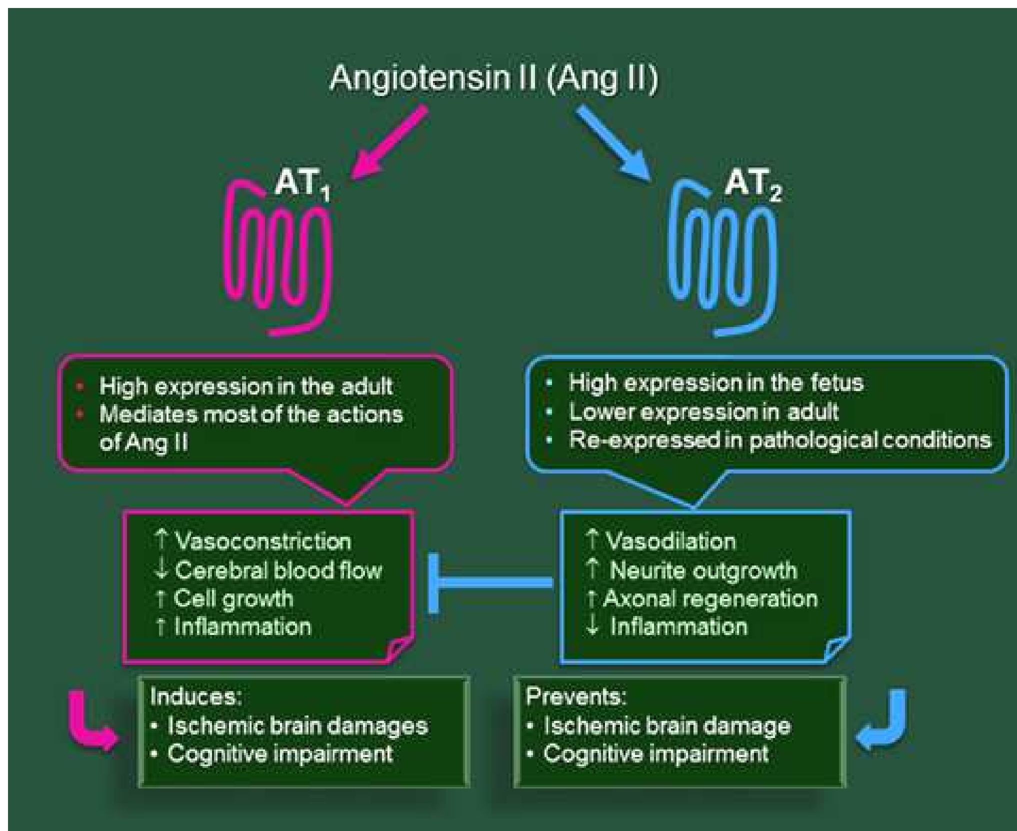


Fig.4- Schematic representation of the effects induced of Ang II by the activation receptor AT1 and AT2

The AT1 receptors (AT1R)

Most of the known physiological effects of ANG II are mediated by angiotensin type 1 receptors (AT1R), which are widely distributed in all organs, including liver, adrenals, brain, lung, kidney, heart, and vasculature. Composed of 359 amino acids, the AT1 receptor (40 kDa) belongs to the seven-membrane superfamily of G protein-coupled receptors. Four cysteine residues are located in the extracellular domain, which represent sites of disulphide bridge formation and are critical tertiary structure determinants. The transmembrane domain and the extracellular loop play an important role in Ang II binding (Hunyady, L 1996).

The binding site for Ang II is different from the binding site for AT1 receptor antagonists, which interacts only with the transmembrane domain

of the receptor (Groblewski, T 1995). Like most G-protein-coupled receptors, the AT1 receptor is also subject to internalization when stimulated by Ang II, a process dependent on specific residues on the cytoplasmic tail (Thomas, W et al 1996). The human AT1R gene has been mapped to chromosome 3. In rats, two isoforms that share 95% amino acid sequence identity have been identified: the AT1AR on chromosome 17 and the AT1BR on chromosome 2 (Griendling et. al, 1996.). AT1A receptors are found predominantly in kidney (Chen X et. al, 1997), lung, liver and vascular smooth muscle, whereas AT1B receptors are expressed mainly in the adrenal and anterior pituitary glands. Once Ang II binds to the AT1R, it activates a series of signalling cascades, which in turn regulate the various physiological effects of Ang II. Evidence shows that when activated by an agonist, AT1Rs couple to $G_{\alpha q/11}$, $G_{\alpha 12/13}$, and $G_{\beta y}$ complexes (Ushio-Fukai M et. al, 1998), which activate downstream effectors including phospholipase C (PLC), phospholipase A_2 (PLA_2), and phospholipase D (PLD). Activation of PLC produces inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) within seconds. IP_3 binds to its receptor on sarcoplasmic reticulum, opening a channel that allows calcium efflux into the cytoplasm. Ca^{2+} binds to calmodulin and activates myosin light chain kinase, which phosphorylates the myosin light chain and enhances the interaction between actin and myosin, causing smooth muscle cell contraction. Activation of phospholipases A_2 and phospholipases D stimulates the release of arachidonic acid, the precursor molecule for the generation of prostaglandins (Griendling et. al, 1996 and Capponi A 1996). Ang II-mediated stimulation of the AT1 receptor coupled to G1/o protein can also inhibit adenylate cyclase in several target tissues, including liver, kidney and adrenal glomerulosa, thereby attenuating the production of the second messenger cAMP (Jard S et. al, 1981; Pobiner B et. al, 1985). cAMP is a vasodilator and when its production is decreased due to AT1 receptor activation,

vasoconstriction ensues. Moreover, the AT1 receptor is also involved in the opening of Ca^{2+} channels and influx of extracellular Ca^{2+} into cells. This mechanism has been linked to Ang II-mediated stimulation of aldosterone production and secretion, as well as vasoconstriction (Apfeldorf, W. J. and Rasmussen, H. 1988). Agonist-AT1R interaction also leads to PLD activation, resulting in hydrolysis of phosphatidylcholine (PC) to choline and phosphatidic acid (PA). PA is rapidly converted to DAG, leading to sustained PKC activation, and sustained muscle contraction. In addition, ANG II can also activate of NAD(P)H oxidases and generation of reactive oxygen species (ROS) (Anja Sachse and Gunter Wolf 2007). AT1R also activates serine/threonine kinases such as PKC and MAPKs that are implicated in cell growth and hypertrophy (Fig.5).

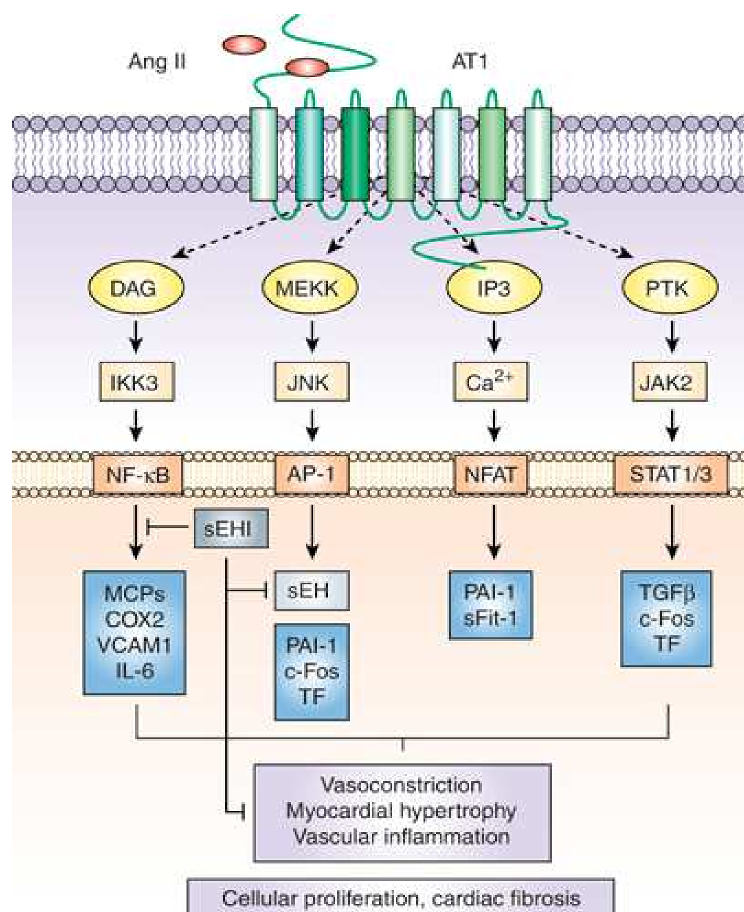


Fig.5- The signal transduction of angiotensin type 1 receptor (AT1) pathways

The AT2 receptors (AT2R)

AT2 receptors, on the other hand, have been suggested to counterbalance most effects that Ang II exerts through the AT1 receptors (Volpe et al. 2003). AT2 receptor functions are related to the inhibition of cell growth, promotion of cell differentiation, and stimulation of apoptosis (Macconi D et. al, 2000). Similarly to AT1 receptor, the AT2 receptor is a seven transmembrane domain receptor (41 kDa), consisting of 363 amino acids, but it is only 34% identical to AT1R. The AT2 receptor gene has been mapped in humans to chromosome X, containing an intronless coding region (Koike G et. al, 1994). AT2R are highly expressed in fetal tissue, including fetal aorta, gastrointestinal mesenchyme, connective tissue, skeletal system, brain, and adrenal medulla. AT2Rs are also expressed at low levels in kidney, lung, and liver, but their exact role in carrying out the functions of ANG II remains undetermined. AT2R have been described to be negatively coupled to guanylate cyclase (inhibition of cGMP production) (Bottari S et. al, 1992) and to activate potassium channels (Kang J et. al, 1993 and 1994). Recently, there have been new insights into AT2 receptor signalling pathways, including activation of protein phosphatases and protein dephosphorylation, the NO-cGMP system, and phospholipase A_2 (release of arachidonic acid). In particular, stimulation of AT2 receptors leads to activation of various phosphatases, such as protein tyrosine phosphatase, MAP kinase phosphatase 1 (MKP-1) (Dzau, V. J. 1997; Fischer T. et. al, 1998), SH2-domain-containing phosphatase 1 (SHP-1) (Bedecs K et. al, 1997 ; Lehtonen J et. al, 1999) and serine-threonine phosphatase 2A (Huang X et. al, 1996; Shenoy U. V et. al, 1999), resulting in the inactivation of extracellular signal regulated kinase (ERK), opening of potassium channels and inhibition of T-type Ca^{2+}channels (Horiuchi M et. al, 1999; Nuoet S. and Nahmias C.2000)(Fig.6).

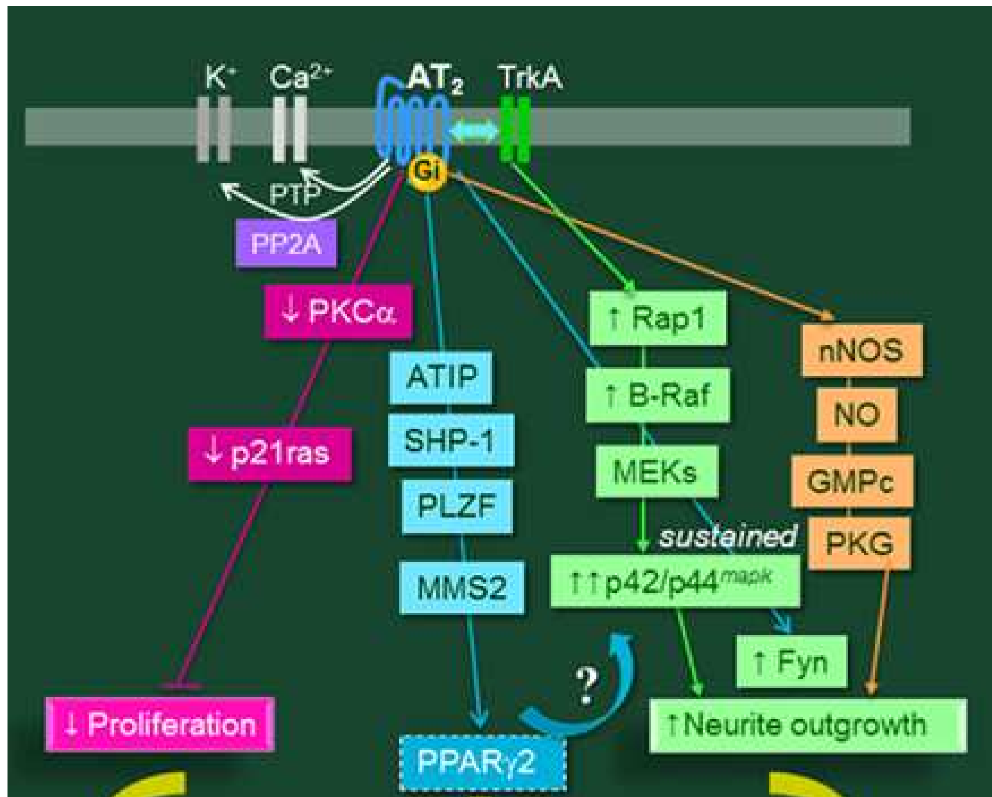


Fig. 6- The signal transduction of angiotensin type 1 receptor (AT1) pathways

Pathological and physiological role of RAS in the GI tract

The hypothesis that Ang II may be involved in the control of various functions of the GI tract was made following the identification of its receptors in the gastrointestinal tract (Fändriks , 2010). Nevertheless, to date the expression of RAS components and receptors for Ang II along the gastrointestinal tract is little studied. The presence of Ang II receptors at various levels along the GI tract has been demonstrated in human, guinea-pig and rat (Wang et al. 2005, Ewert et al. 2006, Spak et al. 2008), suggesting a potential physiological action. Ang II has been reported to regulate intestinal fluid and electrolyte transport (Fandriks 2010, 2011).

In human colon, in addition to the Ang II receptors, also components of the RAS system as the angiotensin-converting enzyme (ACE), appear to be expressed in vessel walls, myofibroblasts, and macrophages in lamina propria, crypt bases, and surface epithelium and (Hirasawa K et. al., 2002). Because intestinal motility has consequent impact on intestinal transit (Nylander 2011), the possibility that Ang II would modulate contractility can be suggested. Recent studies have demonstrated that Ang II may induces contractile responses of longitudinal muscle of guinea -pig small intestine, through the activation of the AT1R localized at the neural level, which mediates release of acetylcholine and substance P , and AT1R localized on smooth muscle cells (Hawcock & Barnes 1993). Subsequently, mRNA and protein for these receptors have been detected at the level of the enteric nervous system of guinea-pigs (Wang et.al, 2005). Indeed, Ang II-induced contractions primarily mediated through AT1 receptors located on the musculature have been pharmacologically characterized in isolated human and rat small intestine (Ewert et al. 2006, Spak et al. 2008) and in human oesophagus (Casselbrant et al. 2007). AT2 receptors, on the other hand, have been suggested to counterbalance most effects that Ang II exerts through the AT1 receptors (Volpe et al. 2003). Even the expression of AT2 receptors has been demonstrated in the gastrointestinal tract of many animal species their role is not yet known (Fandriks, 2010).

Arginine vasopressin (AVP)

The neurohypophysial hormone arginine vasopressin (AVP), which is also known as an antidiuretic hormone, is involved in a wide range of physiological regulatory processes, including renal water reabsorption, cardiovascular homeostasis, hormone secretion from the anterior pituitary and modulation of social behavior and emotional status (Laycock JF. 2010). The nonapeptide hormone AVP, and the structurally related posterior pituitary hormone oxytocin (OT), are synthesized in the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) of the hypothalamus (Armstrong WE. 2004) (Fig.7). Endogenous human vasopressin is also called arginine vasopressin to distinguish it from other members of the vasopressin family, such as lysine vasopressin, found in other mammalian species. It differs in structure from oxytocin by just two amino acids, isoleucine instead of phenylalanine, and leucine instead of arginine.

The gene for vasopressin is situated on chromosome 20, not far from the gene for oxytocin. AVP is secreted into the circulation from the neurohypophysis in response to various factors, including increases in plasma osmolality, emotional stress, emesis, etc., however different studies indicate the existence of peripheral site of production for this hormone as the gut (Fuller PJ et. al, 1985; Friedmann AS et. al, 1993). AVP participates in maintaining body fluid homeostasis by regulating renal water, urea and ion transport, glomerular filtration rate, and renal blood flow (Laycock JF 2010). It is well known that AVP exerts its antidiuretic effect by regulating sodium and water transport via the V2 receptors, which are expressed in the basolateral membrane of the thick ascending limb of Henle’s loop, distal tubules, and the collecting ducts in the kidney (Imbert M et. al, 1975).

Through the V2 receptors, AVP stimulates the Gs protein and adenylate cyclase to increase intracellular cAMP, which stimulates the translocation of AQP2 and the amiloride-sensitive ENaC in the principal cells of the collecting duct, and thereby increases water reabsorption (Inoue T et. al, 2001). AVP could also induce Na^+ reabsorption in the thick ascending limb as well as in the cortical and outer medullary collecting ducts, ensuring the existence of a medullary hypertonic interstitium for maximum water reabsorption (Capurro 2001, Feraille et al. 2003). Vasopressin is synthesized as a large pro-hormone, preprovasopressin, consisting of: an amino-terminal signal peptide; the vasopressin peptide; neurophysin, a carrier protein; and a carboxyl-terminal co-peptide. This prohormone is synthesized principally by the magnocellular neurons of the paraventricular and supraoptic nuclei in the hypothalamus, from where it travels along the supraoptic–hypophyseal tract to the magnocellular presynaptic terminals in the posterior lobe of the pituitary gland. During transport, vasopressin separates from its pro-hormone peptides, and is stored in secretory granules on arrival in the posterior lobe. The carrier protein, neurophysin, is believed to be important in sorting the prohormone into the regulated secretory pathway (de Bree FM & Burbach JP 1998). Once released into the circulation, vasopressin is metabolized rapidly by vasopressinases in the liver and kidney, and has a short half-life of 10–35 min.

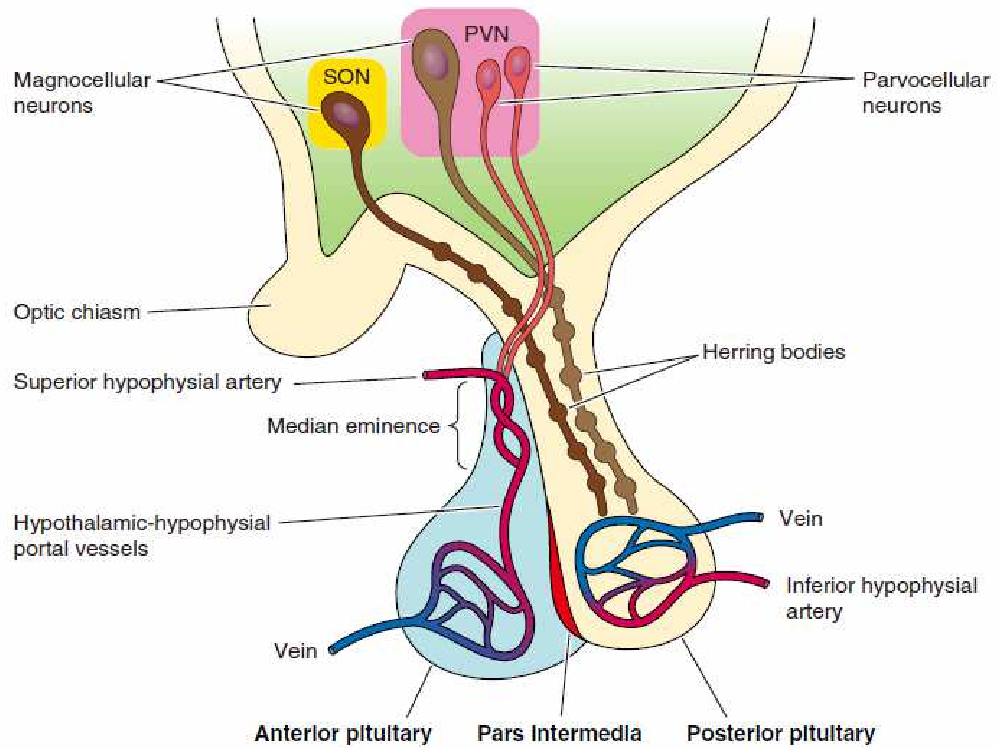


Fig. 7- AVP neurons and hypothalamo-pituitary system.

AVP Receptors and Signal Transduction

The actions of vasopressin are dependent on interaction with receptors that belong to the superfamily of G-protein-coupled receptors. The different vasopressin receptors are closely related, with overall similarity varying from 40 to 85%, and the most conserved regions being the transmembrane a-helices and the first extracellular loop. Three specific vasopressin receptors have been identified: V1, V2 and V3 (Table 1).

Receptor	Key mediated function(s)	Location	Principal second messenger system
V_1	Vasoconstriction, platelet aggregation, glycogenolysis	Vascular smooth muscle, platelets, liver, testes, brainstem	Phosphatidylinositol/calcium
V_2	Water retention	Kidney collecting duct cells	Adenylate cyclase/cAMP
V_3	Corticotropin secretion	Central nervous system	Phosphatidylinositol/calcium
Oxytocin	Vasodilation	Uterine myometrium and endometrium, vascular endothelium	Phosphatidylinositol/calcium
P_2 purinergic	Vasoconstriction	Cardiac endothelium	ATP

Table 1. Vasopressin receptor types, locations and key functions.

The V1 receptors (V1R) are divided into two subclasses, the V1aR and the V1bR. The V1aR mediate vasoconstriction and can be found on smooth muscle cells, but also in liver, kidneys and on platelets. In addition, V1aR are found in a variety of brain nuclei where they have been implicated in the regulation of several social behaviours. The V1bR (also called V3R) are located on the anterior hypophysis as described above (Caldwell HK & Young 2006; Holmes CL et. al, 2003)

The V1 receptors (V1aR)

The V1R (also called V1aR) gene is located on chromosome 12 (region 12q14-15) (Thibonnier M et al, 1996). The V1R are expressed in the liver, vascular smooth muscle cells, testis, blood platelets, adrenal cortex, kidney, spleen, adipocytes, brain (Holmes et al., 2003). AVP binding to the V1R leads to the activation of phospholipases C, D, and A_2 and downstream to multiple pathways; production of inositol 1,4,5-triphosphate and diacylglycerol; simultaneous activation of protein kinase C, p42/p44 MAP kinase, PI_3-kinase, and calcium/calmodulin-dependent kinase II; mobilization of intracellular calcium; influx of extracellular calcium via receptor-operated Ca^{2+} channels and activation of the Na^+-H^+ exchanger (Lange M et. al, 2007; Fleisher-Berkovich S et.al, 2004; Nakatani Y et. al,

2007)(Fig.8). In addition AVP causes vasodilation in some blood vessels, likely via release of NO (Aki Y, et. al, 1994). Other researches have shown that AVP induces vascular endothelial growth factor (VEGF) and proliferation of vascular smooth muscle cells thereby promoting neovascularization (Tahara et. al.1999).

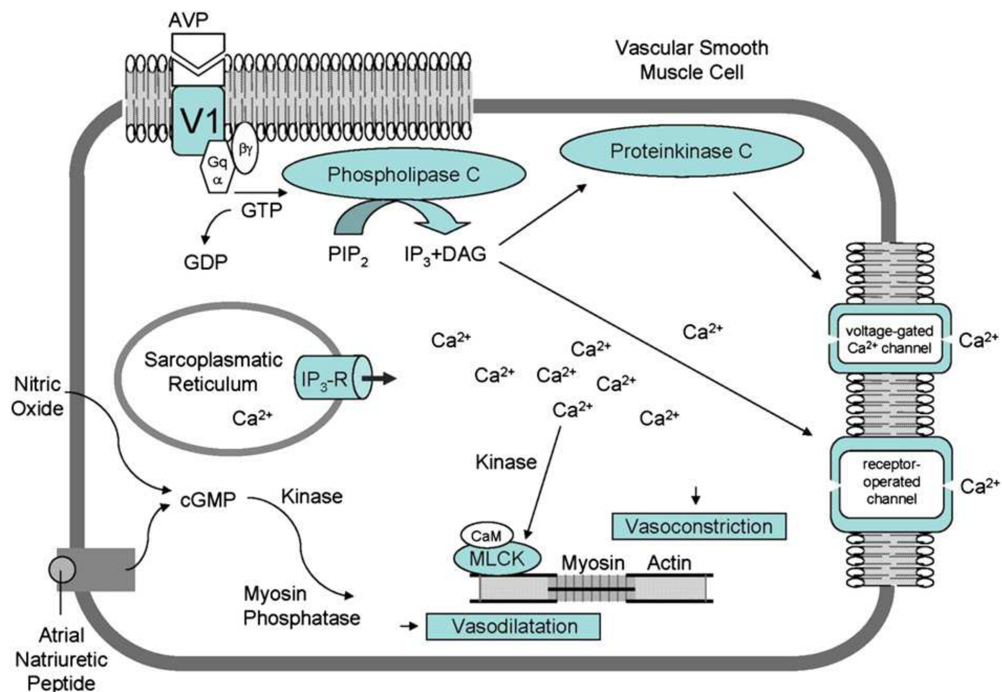


Fig.8- Signal transduction of Vasopressin by V1 receptors.

The V2 receptors (V2R)

The V2R are expressed on the basolateral membrane of the collecting duct in the medullary portion of the kidney, where they mediate the antidiuretic effect of AVP. The human gene encoding for the V2R is located in the chromosome region Xq2818,19. The sequence of the cDNA predicts a polypeptide of 371 amino acids with seven transmembrane domains. The V2R is one of 701 members of the rhodopsin family within the superfamily of GPCRs. AVP binding to the V2R leads to the sequential coupling of the Gs protein, activation of adenylyl cyclase, production of cAMP, and activation of protein kinase A (Fig.9), promoting the insertion of aquaporin

2 water channels (AQP-2) into the luminal surface of the renal collecting tubule cells and later the enhanced synthesis of AQP-2 mRNA and protein (Nielsen et al. 1993, 1995, Fushimi et al.1997).

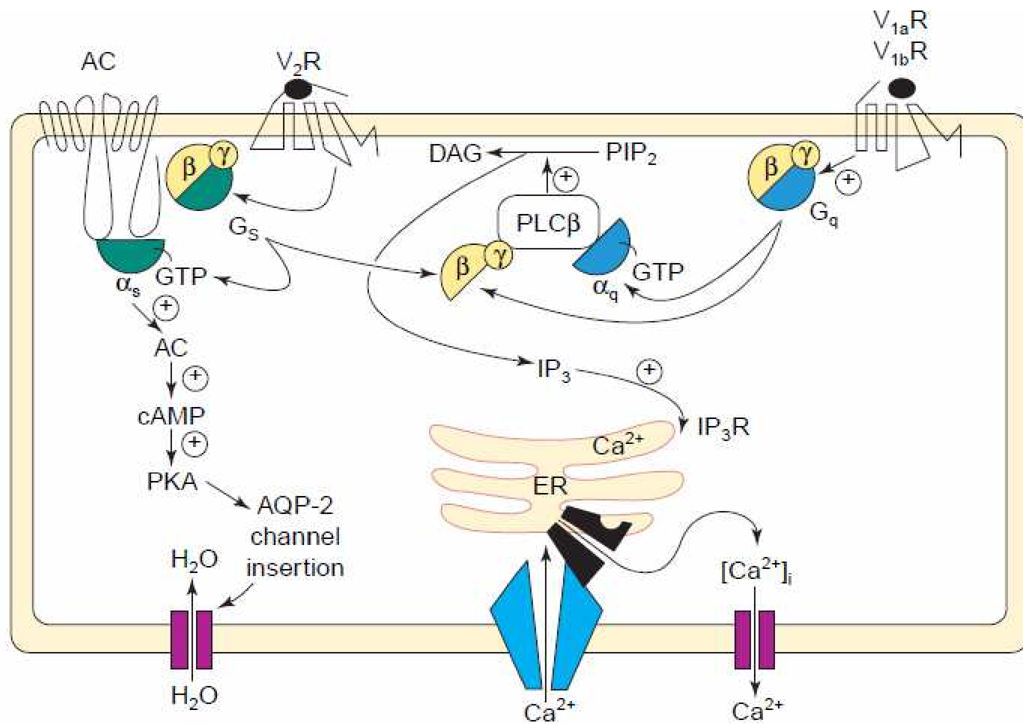


Fig. 9-Coupling mechanisms of the V1 and V2 vasopressin receptors

The V3 receptors (V1bR)

The V3R, called V1bR, are like the other VP receptors, GPCR coupled receptors. The V3R gene is located at the 1q32 region, and it has a sequence of 424 amino acids (Rousseau-Merck MF et. al, 1995). Studies revealed coupling to phospholipase C and adenylyl cyclase, a unique pharmacological profile for this pituitary receptor, distinct from those of the V1R and the V2R subtypes. The V3R were described initially in pituitary cells where it potentiates the release of ACTH. Moreover, recent RT-PCR experiments indicate their presence in other tissues, such as brain, kidney, pancreas, and adrenal medulla (Folny V et al, 2003; de Keyzer Y et. al, 1994).

Vasopressin in the Gastrointestinal Tract

AVP has been reported to regulate electrolyte and water transport in the colon both in vivo and in vitro. In vivo, in the rat and human colon AVP inhibits Na^+ and Cl^- absorption (Dennhardt R et. al, 1979; Levitan R et. al, 1968). By the contrast, AVP stimulates in vitro $NaCl^-$ and water absorption, as well as inhibits Cl^- secretion in mouse, rat and human colon (Bridges RJ et. al, 1983 and 1984; Knobloch SF et. al, 1989; Mobasheri A et. al, 2005; Grady GF et. al, 1970; Vincentini-Paulino 1992). Unclear and conflicting are the studies about a possible effect of AVP in the control of gastrointestinal motility. Voderholzer WA et. al, (1995) suggested that AVP at physiological level might not influence colonic motility in rats and humans.

Ward et. al, (1997) reported that AVP at low concentration increased slow wave activity and phasic contraction in the muscle strips from dog colon, whilst at higher concentration it caused an inhibitory effect. Indeed AVP can be produced locally in cells of human and rat gastrointestinal system (Friedmann AS et. al, 1991) and AVP receptors are reported to be expressed throughout gastrointestinal tract (Monstein HJ et. al, 2008). V1a receptors has been found on the neurons of myenteric plexus in rat stomach, where AVP has been suggested to act as neuromodulator of enteric cholinergic neurons inducing excitatory effects on the contractility (Junfang Qin et. al, 2009). Recently AVP was shown to inhibit the contractions through production of nitric oxide in strips of the circular muscle of rat colon (Jing H et. al, 2011). All together these data would suggest that AVP may be one of the brain–gut peptides influencing gastrointestinal functions also acting at peripheral level and that the effects of vasopressin can be different in the various parts of the GI tract and among species.

Capitolo 2

Materials and methods

Animal tissue

All animal procedures were in conformity with the Italian D.L. no. 116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609/ECC). Experiments were performed on adult male mice (C57BL/10SnJ; weighing 25.5± 0.5 g; Charles River Laboratories, Calco-Lecco, Italy), under controlled conditions (12/12 light/dark cycle; ambient temperature 22 ± 1 °C; humidity 55 ± 5%) and they had free access to tap water and standard pellet food. Experimental procedures were approved by Ministero della Sanità (Rome, Italy). Mice were euthanized using isoflurane anesthesia followed by cervical dislocation , and after a midline laparotomy, the entire colon was removed (about 15 cm length) and placed in pre-oxygenated Krebs solution to obtain circular or longitudinal muscle strips (20 mm in length) of mouse proximal (just after the caecum) or distal (about 5 mm proximal to the anus) colon. Some specimen were frozen and stored at -80˚C for subsequent biomolecular analysis.

Human tissue

Specimens of human sigma colon were obtained from 28 patients (aged 60-85, 14 females and 14 males) with no symptoms of major clinical motility disorders, and who underwent surgery for neoplastic conditions at the Mediterranean Institute for Transplantation and Advanced Specialized Therapies (ISMETT), in Palermo, Italy. The experimental protocol was approved by the institutional ethics committee, and written informed consent

was obtained from all individuals prior to surgery. Samples consisted of whole wall sections of the colon from a macroscopically normal region taken at a distance of at least 5 cm from any visible lesion. For the mechanical experiments, the colonic specimens (n =5) were immediately placed in pre-oxygenated Krebs solution in a dissection dish in order to remove the mucosal layer and stored overnight at 4 °C. Strips of longitudinal muscle (1 cm in length, 2-3 mm thick) were obtained. Other samples (n =3) were frozen and stored at -80˚C for subsequent biomolecular analysis.

Recording of mechanical activity

The following experimental approach was chosen to study the muscle function under conditions where the influence of external factors is removed, but the muscle itself performs in a manner analogous to its in vivo capacity. Either longitudinal or circular muscle strips of mouse or human colon were suspended in a four-channel organ bath (Fig.1).

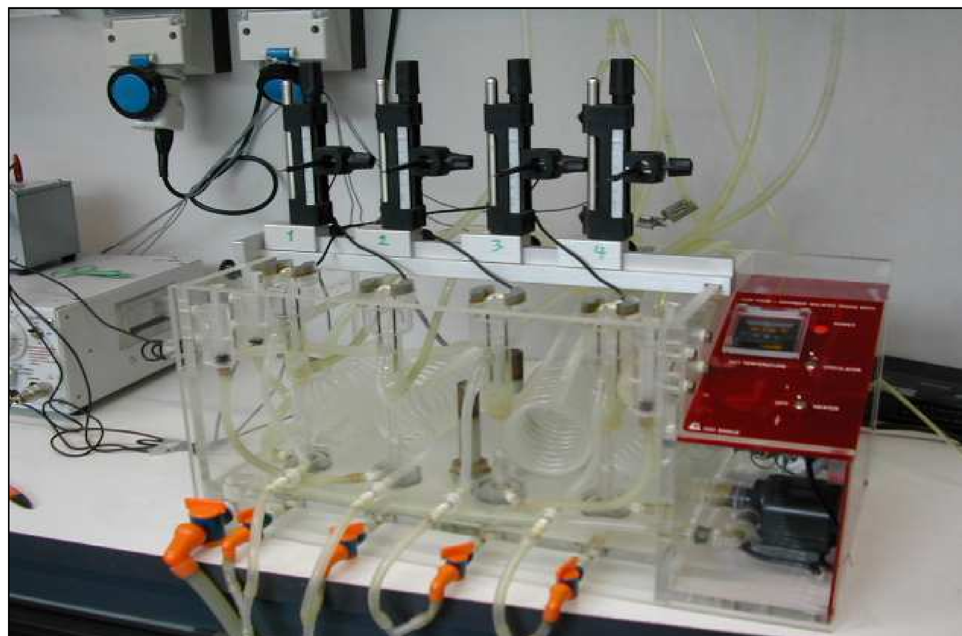


Fig. 1: Organ bath system

The distal end of each strip was tied to an organ holder and the proximal end was secured with a silk thread to an isometric force transducer (FORT 25, Ugo Basile, Biological Research Apparatus, Comerio, VA, Italy). Mechanical activity was digitized on an A/D converter, visualized, recorded and analyzed on a personal computer using the PowerLab/400 system (Ugo Basile, Italy). Mouse preparations were subjected to an initial tension of 200 mg and were allowed to equilibrate for at least 30 min, whilst human preparations were subjected to an initial tension of 1 g and allowed to equilibrate for 2h, to develop stable spontaneous tone. Rhythmic spontaneous contractions of varying amplitude developed in all preparations.

Solution and drugs

Krebs solution consisted of (mM): NaCl=119; KCl=4.5; $MgSO_4$ = 2.5; $NaHCO_3$ = 25; KH_2PO_4 = 1.2; $CaCl_2$ = 2.5; and glucose = 11.1. Drugs used were atropine sulphate, carbamylcholine chloride (carbachol, CCh), hexamethonium bromide, isoproterenol, ondansetron hydrochloride dehydrate and TTX from Sigma-Aldrich Inc. (St Louis, MO, USA); angiotensin II, 1-[[4-(Dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c] pyridine-6-carboxylic acid ditrifluoroacetate (PD123319) and 2-Butyl-4-chloro-1-[[2′-(1Htetrazol-5-yl)-[1,1′-biphenyl]-4-yl]methyl]-1H-imidazole-5-methanol potassium salt (losartan) from Tocris Bioscience (Bristol, UK); (S)-N-methyl-N[4-(4-acetylamino-4-phenylpiperi-dino)-2-(3,4-dichloro-phenyl) butyl] benzamide (SR48968) and (S)-1-[2-[3-(3,4-dichlorphenyl)-1 (3-isopropoxy-phenylacetyl)piperidin-3yl]ethyl]-4-phenyl-1 azaniabicyclo[2.2.2] octane chloride (SR140333) were gifts from Sanofi Recherche (Montpellier Cedex, France); [β-Ala8]-NKA(4−10) and [Sar9,

Met (O2)11]-substance P was from Calbiochem-Novabiochem (Laufelfingen, Switzerland); cyclopiazonic acid (CPA), 2′,3′-dideoxyadenosine (DDA), ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid tetrasodium salt (EGTA), indomethacin, nifedipine, [deamino-Pen1, O-Me- Tyr2, Arg8]-vasopressin (V-1880) (all purchased from Sigma-Aldrich, Inc., St. Louis, USA); arginine vasopressin (AVP) and oxytocin (Tocris Bioscience, Bristol, UK); {1-[6((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione} (U-73122) (Calbiochem, Darmstadt, Germany). Indomethacin solution was prepared fresh on the day of experiment in 2% sodium carbonate solution and the pH was adjusted to 7.4. U-73122 and DDA were dissolved in dimethyl sulfoxide and nifedipine was dissolved in ethanol and further diluted in Krebs. SR48968 and SR140333 were dissolved in dimethyl sulphoxide (0.1% final concentration), [Sar9, Met (O2)11]-substance P was dissolved in diluted acetic acid while [β-Ala8]-NKA(4–10), dissolved in diluted ammonia. All the other drugs were dissolved in distilled water. Working solutions were then dissolved in Krebs solution.

Statistical analysis

All data are presented as means ± SEM: ‘n’ indicates the number of animal preparations. Contractile responses induced by Ang II and by AVP were reported as a percentage of the effect induced by 10 µM CCh. Ang II and AVP responses were fitted to sigmoid curves (Prism 4.0,Graph-PAD, San Diego, CA, USA), and EC50 values with 95% confidence limits (CLs) were determined. Antagonist potency was expressed as the negative logarithm of the concentration of the antagonist required to cause a twofold rightward shift of the agonist dose –response curve (pA2 value), calculated by nonlinear regression analysis of the individual dose–response curves.

Statistically significant differences were calculated by Student's t-test or by analysis of variance followed by Bonferroni's test. $P < 0.05$ was considered statistically significant.

RNA preparation and RT-PCR analysis in preparation of mouse colon

Total RNA was extracted from whole thickness proximal and distal colon and in a preparation devoid of mucosa layer, using PureLink™ RNA Mini Kit (Invitrogen, Paisley, UK) according to the manufacturer's instructions. After quantification by spectrophotometry, 1 mg of total RNA was reverse-transcribed in a final volume of 50 mL using the High Capacity c-DNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and the following thermal cycle profile: 10 min at 25°C, 2 h at 37°C and 5 min at 85°C as described by the kit. The oligonucleotide primers for amplification of cDNA (30 ng per reaction) encoding for angiotensin II type 1 subtype A and B receptors ($AT1_A$, $AT1_B$), angiotensin II type 2 receptor (AT2), angiotensinogen (AGT), angiotensin-converting enzyme (ACE), renin and b-actin were designed from published mouse cDNA sequences and are summarized in Table 1. PCR analysis was performed in triplicate. Each PCR cycle consisted of denaturing at 94 °C for 45 s, annealing at 56 °C (AT_{1A} receptor, AGT and b-actin) for 60 s or at 48 °C (AT_{1B} receptor and ACE) for 45 s or 58 °C (renin) for 45 s or 40 °C (AT_2 receptor) for 60 s and extension at 72 °C for 1 min. This was repeated for 35 cycles, followed by extension at 72 °C for 15 min. The amplimers were separated on a 1.8% agarose gel containing 0.5 µg ml-1 of GelStar Nucleic Acid Gel Stain (Lonza Rockland, ME USA) for visualization.

RAS component	Primer sequences	Fragment size
Angiotensinogen (AGT)	FORWARD: 5-TATCCACTGACCCAGTTCTTT- 3 REVERSE: 5-AGTGAACGTAGGTGTTGAAA-3	133 bp
RENIN	FORWARD: 5'-ATGAAGGGGGTGTCTGTGGGGTC-3' REVERSE: 5'-ATGTCGGGGAGGGTGGGCACCTG-3'	194 bp
ACE	FORWARD: 5'-CTGCGTAGAGGTGCCAACC-3' REVERSE: 5'-ACGGTGTCACGTTTGGGATG-3'	357 bp
AT_{1A}	FORWARD: 5-TCACCTGCATCATCATCTGG-3 REVERSE: 5-AGCTGGTAAGAATGATTAGG-3	204 bp
AT_{1B}	FORWARD: 5-TGGCTTGGCTAGTTTGCCG-3 REVERSE: 5-ACCCAGTCCAATGGGGAGT-3	121 bp
AT_2	FORWARD: 5’-TCCTTTTGATAATCTCAAC-3’ REVERSE: 5'-CAAACACTTTGCACATCACA-3'	310 bp
β-ACTIN	FORWARD: 5'-CCGCCCTAGGCACCAGGGT-3' REVERSE: 5'-GGCTGGGGTGTTGAAGGTCTCAAA-3'	300 bp

Table 1-Primer sequences for reverse transcription–polymerase chain reaction (RT-PCR)

RNA preparation and RT-PCR analysis
in preparation of human colon

Total RNA was extracted from whole thickness SIGMA colon of human using PureLink™ RNA Mini Kit (Invitrogen, Paisley, UK) according to the manufacturer’s instructions. The method has been described previously with some modification. After quantification by spectrophotometry, 1 µg of total RNA was reverse-transcribed in a final volume of 50 µl using the High

Capacity c-DNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and the following thermal cycle profile: 10 min at 25°C, 2 h at 37°C and 5 min at 85°C (as described by the kit). cDNA (30 ng per reaction) was denatured and subjected to PCR amplification. The oligonucleotide primers were based on available cDNA sequences for human angiotensin II type subtype A (AT1), angiotensin II type 2 receptor (AT2), angiotensinogen(AGT), angiotensin-converting enzyme (ACE), renin and β-actin. These primers are reported in the study of Koki et al, 2002. PCR analysis was performed in duplicate. Each PCR cycle consisted of denaturing at 94°C for 1min , annealing 55°C for 1 min (AT1 receptor and rennin); or at 53°C (AT2 receptor, ACE and β-actin) and 57°C (Agt), and extension at 72°C for 1 min. This was repeated for 35 cycles, followed by extension at 72°C for 15 min. The amplimers were separated on a 1.8 % agarose gel containing 0.5 µg ml-1 of GelStar Nucleic Acid Gel Stain (Lonza Rockland, ME USA) for visualization, and the gel was scanned under under UV light.

Capitolo 3

Angiotensin II contractile effects in mouse and human colon: role for AT_1 receptors

Rationale and aims

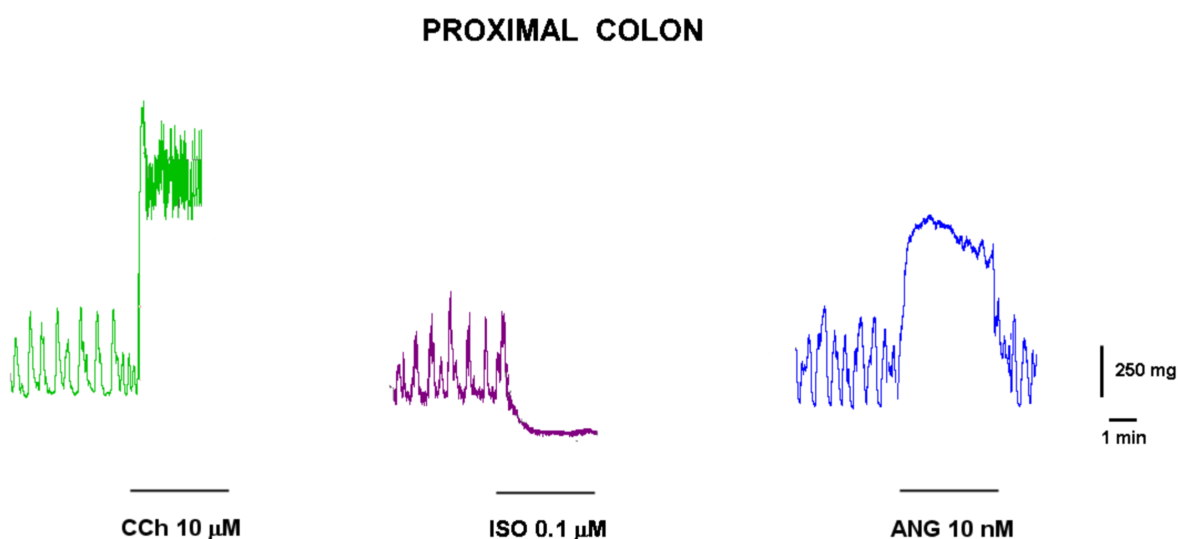
The control of fluid and electrolyte balance is closely connected with gastrointestinal function which acts in concert with the kidneys in enterorenal axis. In particular the colon plays a decisive role in the reabsorption of water and other electrolytes, and hormones involved in these important processes of absorption, seem to be of the "brain-gut peptides" can also affect gastrointestinal motility and interact with the enteric nervous system (ENS). Ang II has been demonstrated to be able to modulate the processes of absorption/secretion of ions and water at physiological concentration (Fandriks 2010, 2011). However, unclear and conflicting are the studies about possible effects of Ang II in the control of gastrointestinal motility (see introduction). Therefore, in consideration that the role of this hormone in the regulation of the motor activity of the gastrointestinal tract (GI) is far from being clear, the aim of this study was to analyse, *in vitro,* the possible effects of Ang II on the contractility colonic motor activity in mouse and human and to identify the receptor subtypes and the related action mechanisms underlying the observed effects. Moreover, through molecular biology techniques, the expression of transcripts of the main components of the RAS system will be evaluated in the intestinal specimen.

Results:

Ang II in mouse colon

Effects of Ang II on motor activity

Isolated segments of mouse colon displayed spontaneous activity consisting of phasic contractions with an amplitude of 460.1 ± 41.0 mg and a frequency of 5.30 ± 0.38 c.p.m. in the proximal region (n=25), and with an amplitude of 244.2 ± 0.8 mg and a frequency of 5.2 ± 1.5 c.p.m. in the distal region (n=28). Ang II (0.001-100 nM) caused a concentration-dependent contractile effect on the longitudinal muscle in both proximal and distal colon, which persisted throughout the application of the drug (Fig. 1-3). The maximal response in both regions was observed at the dose 10 nM (absolute increase in muscular tone was 958.5 ± 15.0 mg (n=12) in proximal and 765.5 ± 18.2 mg (n=12) in distal colon).



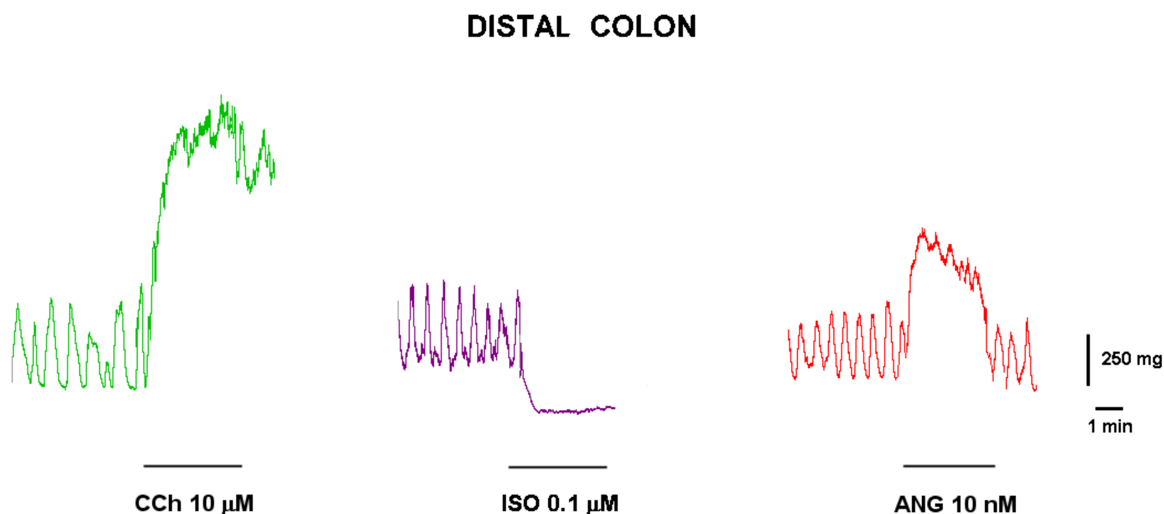
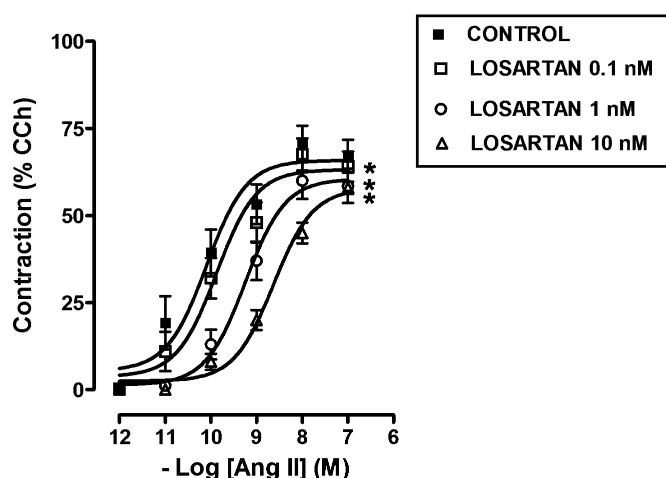


Fig. 1- Original recordings showing the mechanical responses evoked by CCh, isoproterenol (ISO) or Ang II in mouse proximal and distal colon

Analysis of the dose-response curves for the increase in the basal tone pointed out that Ang II shows a similar potency in the two different preparations (proximal colon: EC_{50}: 0.08 nM, 95% Cls 0.03- 0.23 nM, n= 12; distal colon: EC_{50} = 0.05 nM, 95% Cls 0.02- 0.09 nM, n= 12) (Fig. 2). We never observed an inhibitory/relaxant effect induced by Ang II at any concentration tested. In both proximal and distal colon, the responses to Ang II were antagonized by losartan, AT1 receptor antagonist, in a concentration-dependent fashion (pA_2= 9.8 ± 0.05 and 10.1±0.02) (Fig. 2).

PROXIMAL COLON **DISTAL COLON**



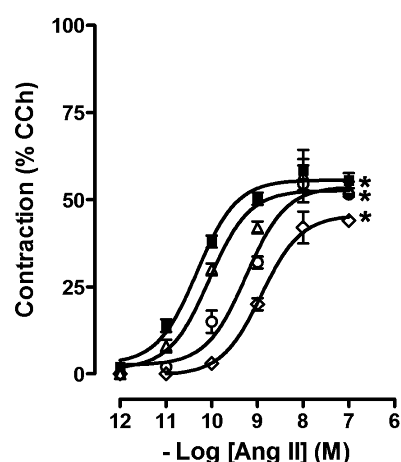


Fig. 2 Concentration–response curves to Ang II before and after different concentrations of losartan, AT1 receptor antagonist (n = 4 each), in mouse proximal and distal colon. Data are means ± SEM and are expressed as percentage of the maximal effect induced by 10 μM CCh. The values for the control curves are the means of the control data obtained before each treatment (n = 12 for both proximal and distal colon). * $P < 0.05$ when the concentration–response curves were compared to those obtained in the respective control condition.

Losartan *per se* had no effects on the spontaneous activity. On the contrary, PD123319 (up to 0.1 μM), AT2 receptor antagonist, was without any effect (Fig. 3). The combination of losartan (10 nM) plus PD123319 (0.1 μM) caused no change of the Ang II-induced contractile effect than losartan alone (Fig. 3).

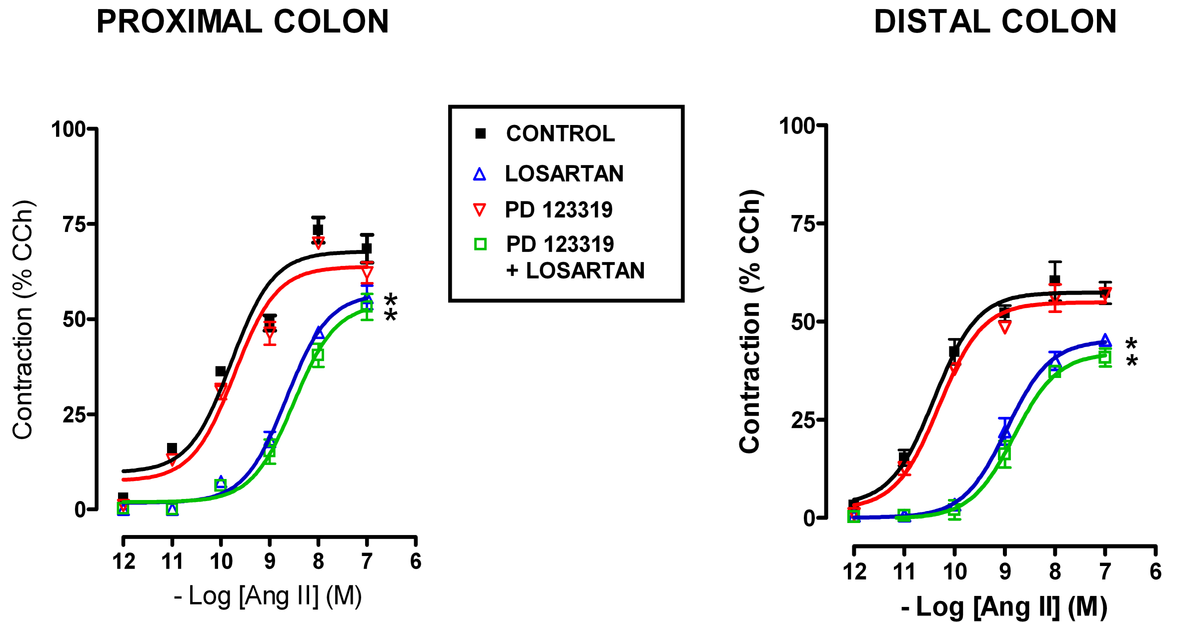
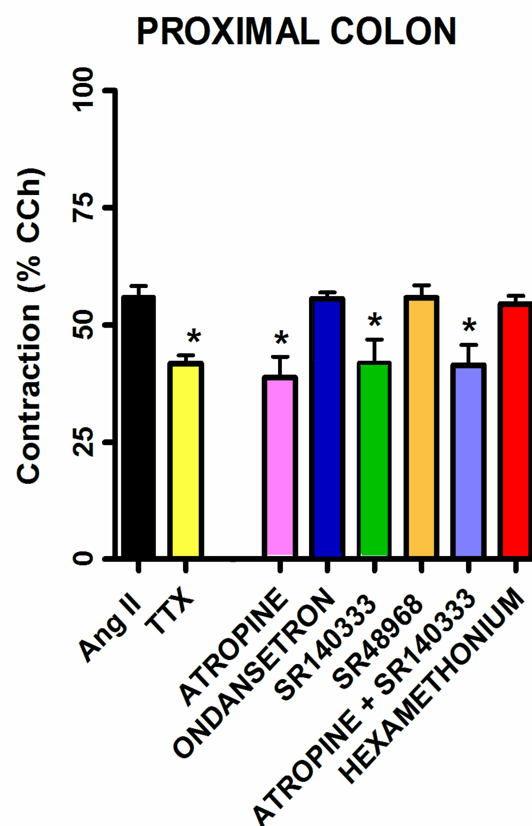


Fig. 3 - Concentration-response curves to Ang II before and after PD123319 (0.1 μM, n=3), AT2 receptor antagonist, losartan (10 nM, n=3), AT1 receptor antagonist, or PD123319 plus losartan (n=3) in mouse proximal and distal colon. Data are means ± s.e.m and are expressed as percentage of the maximal effect induced by 10 μM CCh. The values for the control curves are the means of the control data obtained before each treatment (n=9 for both proximal and distal colon).* $P<0.05$ when the concentration-response curves were compared to those obtained in the respective control condition.

TTX (1 μM), Na^+ voltage-gated neural channel blocker, partially reduced the contractile response to a submaximal dose of Ang II (1 nM) in the proximal colon, whilst the response was abolished in the distal colon (Fig. 4). To characterize the neural pathway(s) mediating the indirect (TTX-sensitive) contractile effect induced by activation of AT1 receptors, Ang II was tested in the presence of antagonists for cholinergic, serotoninergic or tachykininergic receptors, the main excitatory system involved in the control gut motility.

In proximal and distal colon, atropine (1 μM), muscarinic receptor antagonist, or SR140333 (0.1 μM), NK1 receptor antagonist, reduced the excitatory effects induced by Ang II (1 nM) (Fig. 4). On the contrary, ondansetron (0.1 μM), 5-HT_3 receptor antagonist, SR48968 (0.1 μM), NK2 receptor antagonist, or hexamethonium (30 μM), nicotinic receptor antagonist, were ineffective (Fig. 4). Multiple comparisons showed that there was no difference in the antagonism of Ang II-contractile effect by TTX, atropine and SR140333. Moreover, the joint application of atropine (1 μM) and SR 140333 (0.1 μM) did not produce any additive effect (Fig. 4). None of the antagonists used had any significant influence on the amplitude and frequency of the spontaneous contractile activity.



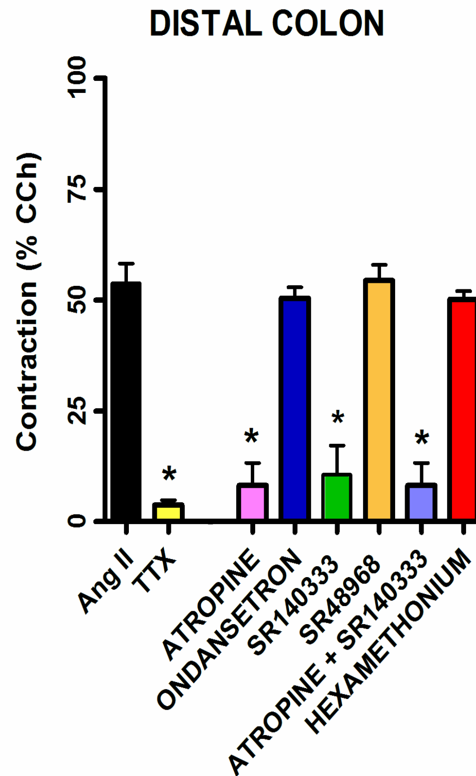


Fig. 4 - Histogram showing the effects of Ang II (1 nM) in mouse proximal and distal colon in the absence or in the presence of the Na^+ voltage-gated neural channel blocker ,TTX (1 µM, n=5), the muscarinic receptor antagonist, atropine (1 µM, n=4), the 5-HT_3 receptor antagonist, ondansetron (0.1 µM, n=3), the NK1 receptor antagonist, SR140333 (0.1 µM, n=4), the NK2 receptor antagonist, SR48968 (0.1 µM, n=3) or the nicotinic receptor antagonist, hexamethonium (30 µM, n=3). Data are means ± s.e.m and are expressed as percentage of the maximal effect induced by 10 µM CCh. The graphed value for the control bar is the mean of the control data obtained before each treatment. *P<0.05 when compared to the respective own control condition.

Lastly, as shown in Fig. 5, atropine (1 µM) significantly reduced the contraction to a specific NK_1 receptor agonist, [Sar^9, $Met(O_2)^{11}$]-substance P(1 µM), whilst SR140333 (0.1 µM) failed to affect the contraction evoked by CCh (10 µM) in distal colon. Same results were obtained in the proximal colon.

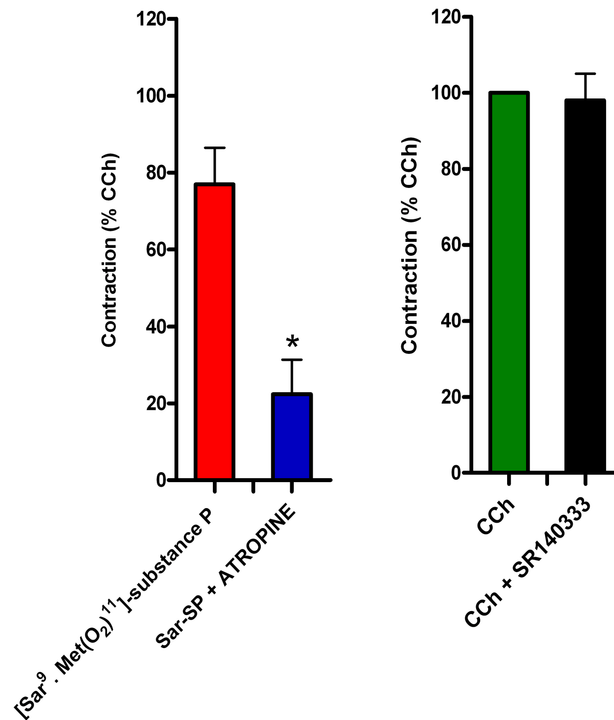


Fig. 5- Histogram showing the effects of the NK1 receptor agonist [Sar9, Met(O2)11]-substance P (1 μM) in the absence or in the presence of the muscarinic receptor antagonist, atropine (1 μM, n = 3), and of CCh (10 μM) in the absence or in the presence of the NK1 receptor antagonist, SR140333 (0.1 μM, n = 3) in mouse distal colon. Data are means ±SEM and are expressed as percentage of the maximal effect induced by 10 μM CCh. *P < 0.05 when compared to the respective own control condition.

Transcripts encoding RAS components in proximal and distal colon

RT-PCR was used to investigate the expression of mRNA encoding for renin, angiotensinogen (AGT), angiotensin-converting enzyme (ACE), angiotensin receptors (AT1A, AT1B, AT2) in proximal and distal colon preparation. Transcripts encoding renin, AGT, ACE, and AT1A receptors were found in the whole thickness preparations (Fig. 6).

AT1B mRNA was not expressed in both preparations, while AT2 mRNA was expressed just in the distal colon (Fig. 6).

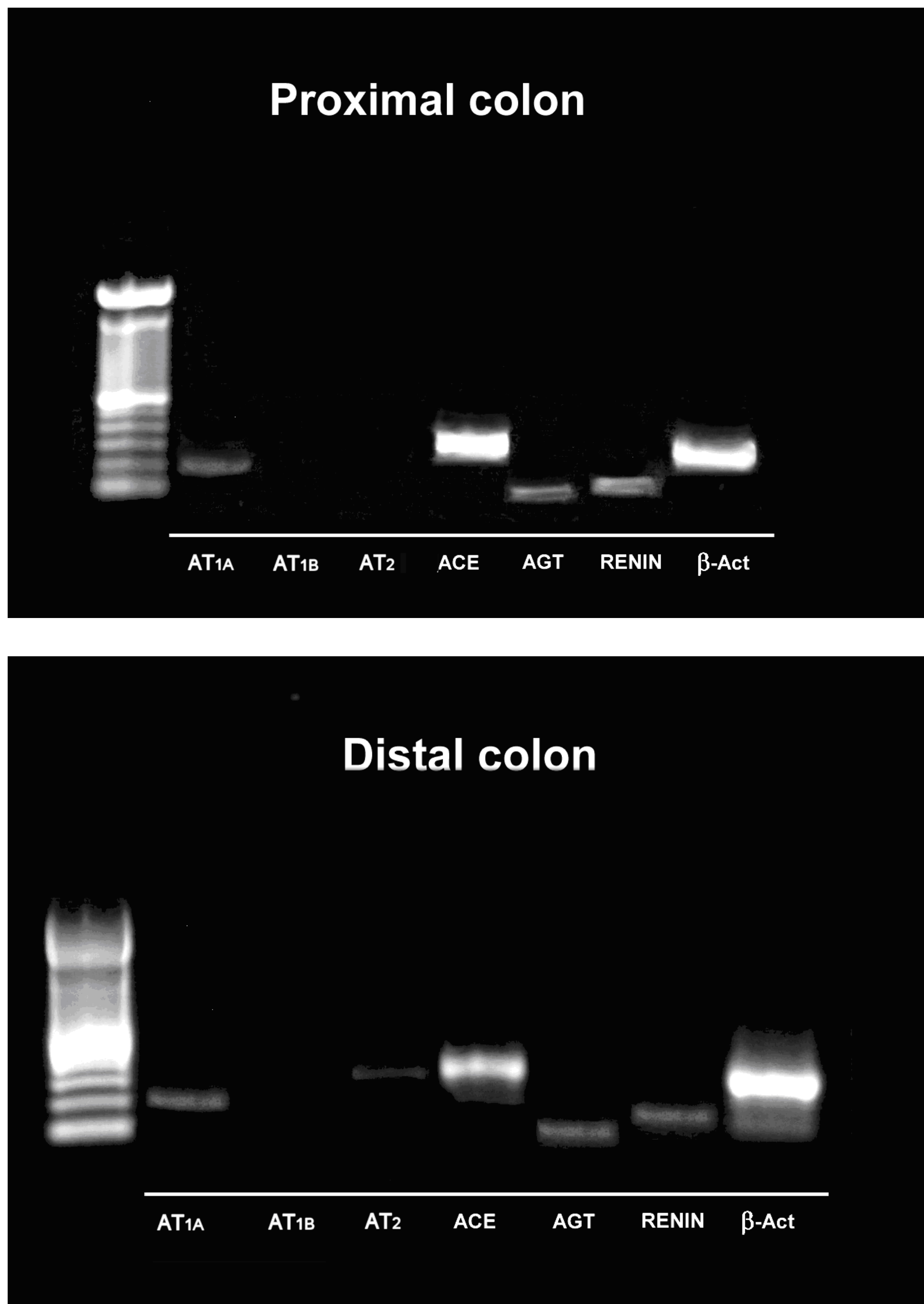


Fig. 6 - Expression of transcripts encoding the components of RAS, renin, angiotensinogen (AGT), angiotensin-converting enzyme (ACE), angiotensin receptors (AT1A, AT1B, AT2) in proximal and distal colon preparation. β-Actin (β-Act) primer was used as a control for cDNA integrity. A 100 bp DNA ladder was used as marker.

Ang II in human colon

Transcripts encoding RAS components in proximal and distal colon

RT-PCR was used to investigate the expression of mRNA encoding for RAS component renin, angiotensinogen (AGT), and receptors (AT1, AT2) in human SIGMA colon preparation. Transcripts encoding for AT1 and AT2 angiotensin receptor subtypes and for angiotensin-converting enzyme (ACE) were expressed in the whole thickness preparations, while we did not found expression of mRNA for angiotensinogen (AGT) and for renin.

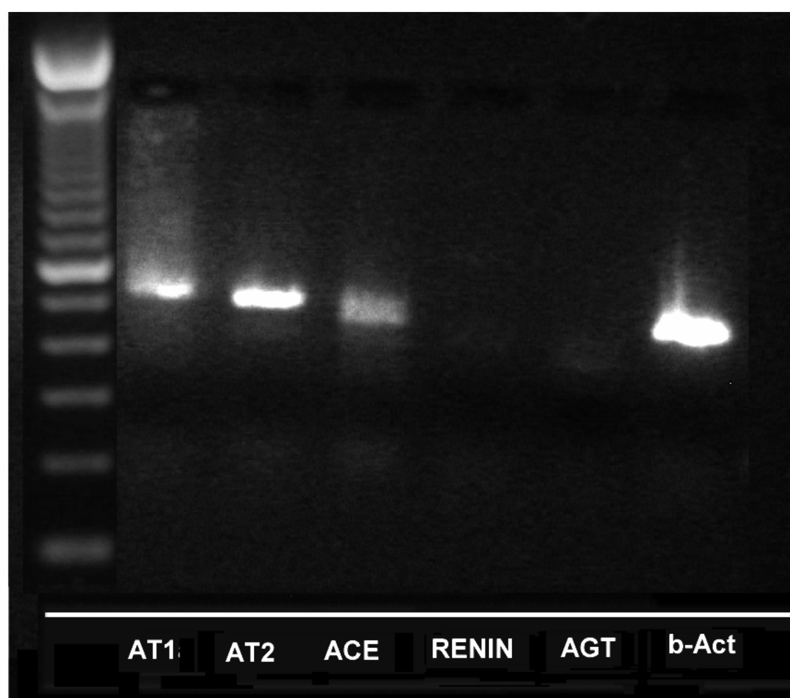


Fig.1 - Expression of transcripts encoding the components of RAS, renin, angiotensinogen (AGT), angiotensin-converting enzyme (ACE), angiotensin receptors (AT1, AT2) in human colon preparation. β-Actin (β-Act) primer was used as a control for cDNA integrity. A 100 bp DNA ladder was used as marker.

Effects of Ang II on motor activity

Isolated circular muscular strips of human sigma colon displayed spontaneous activity consisting of phasic contractions with an amplitude of 2.0 ± 0.27 g and a frequency of 3.20 ± 0.28 c.p.m. (n= 5). Ang II (0.1-100 nM) caused a concentration-dependent contractile effect on circular muscle in distal colon (EC50 = 0.2 nM, 95% Cls 0.09-0.3 nM, n= 5) (Fig. 2-3).

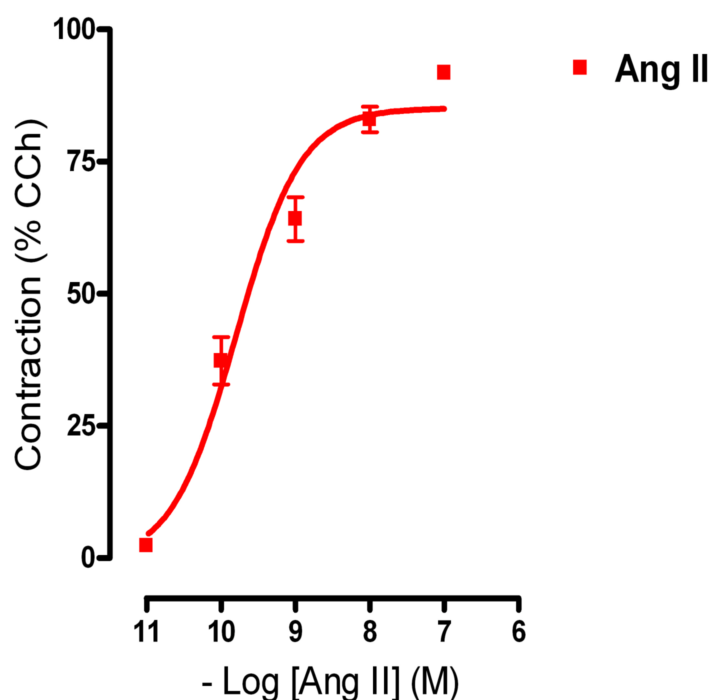


Fig.2- Concentration-response curves to Ang II (0.1-100 nM) in circular muscular strips of human sigma colon. Data are means ± SEM and are expressed as percentage of the maximal effect induced by 10 μM CCh. The values for the control curves are the means of the control data obtained before each treatment (n =5). * $P < 0.05$ when the concentration–response curves were compared to those obtained in the respective control condition.

The effect persisted throughout the application of the drug and was reversible after washout. The maximal response to Ang II was observed at the dose of 100 nM (absolute increase in muscular tone was 3.36 ± 0.50 g ; n=5) (Fig.3).

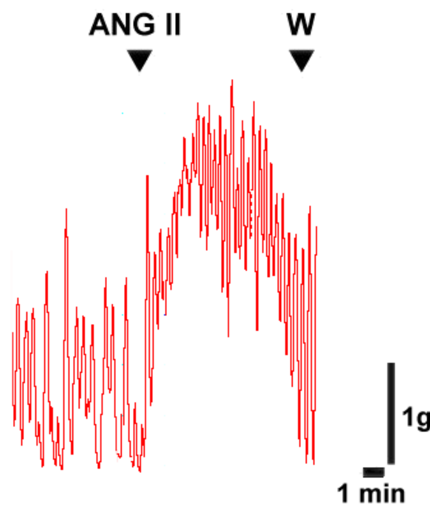


Fig.3- Original recordings showing the mechanical responses evoked Ang II in human colon.

The responses to Ang II was antagonized by losartan (10 nM, n=5), AT1 receptor antagonist, which per se did not modify spontaneous activity. On the contrary, PD123319 (10 nM, n=5), AT2 receptor antagonist, did not affect the response to Ang II. The joint application of losartan (10 nM) and PD123319 (10 nM) did not produce any additive effect (Fig. 4).

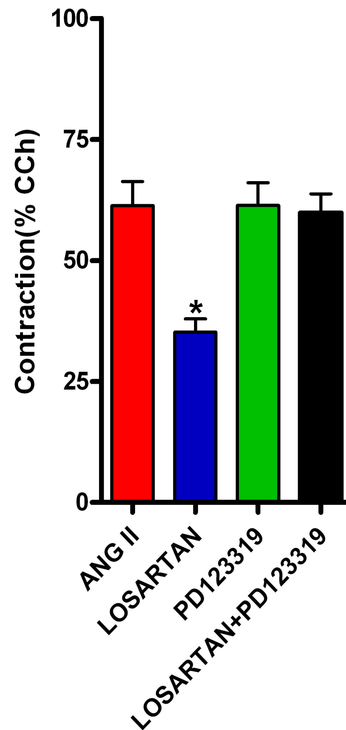


Fig.4- Histogram showing the effects of Ang II (10 nM) in human colon before and after of losartan (10 nM, n=5), AT1 receptor antagonist, PD123319 (10 nM, n=5), AT2 receptor antagonist or PD123319 plus losartan (n=5). Data are means ± s.e.m and are expressed as percentage of the maximal effect induced by 10 µM CCh. The graphed value for the control bar is the mean of the control data obtained before each treatment. *P<0.05 when compared to the respective own control condition.

TTX (1 µM), Na^+ voltage-gated neural channel blocker, partially reduced the contractile response to a submaximal dose of Ang II (10 nM) (Fig. 5). To characterize the neural pathway(s) mediating the indirect (TTX-sensitive) contractile effect induced by activation of AT1 receptors, Ang II was tested in the presence of atropine, cholinergic muscarinic receptors antagonist, or SR48968, tachykininergic NK2 receptor antagonist, affecting the main excitatory systems involved in the control gut motility.

Atropine per se decreased the amplitude and frequency of the spontaneous contractile activity, whilst SR48968 only for amplitude. Indeed only SR48968 (0.1 μM) was able to reduce significantly the excitatory effects induced by Ang II (10 nM), being atropine (1 μM) ineffective (Fig.5). Lastly, the contraction to the specific NK2 receptor agonist, β-Ala (1 μM), was not affected by TTX (1 μM) or atropine (1 μM) (Fig.6).

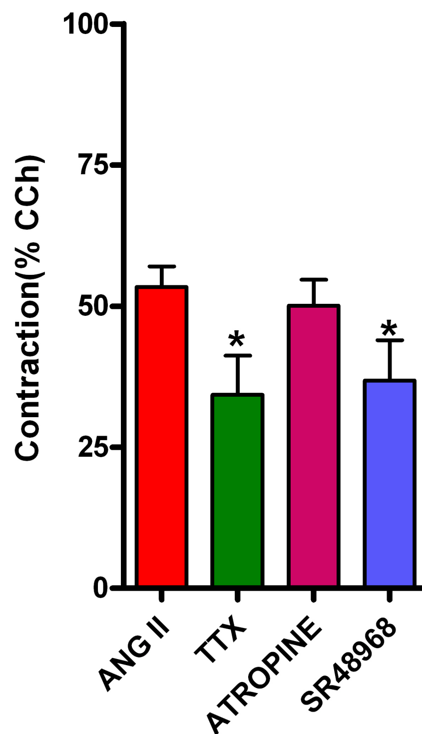


Fig. 4 - Histogram showing the effects of Ang II (10 nM) in human colon in the absence or in the presence of the Na^+ voltage-gated neural channel blocker, TTX (1 μM, n=5), the muscarinic receptor antagonist, atropine (1 μM, n=5) and the NK2 receptor antagonist, SR48968 (0.1 μM, n=5). Data are means ± s.e.m and are expressed as percentage of the maximal effect induced by 10 μM CCh. The graphed value for the control bar is the mean of the control data obtained before each treatment. *P<0.05 when compared to the respective own control condition.

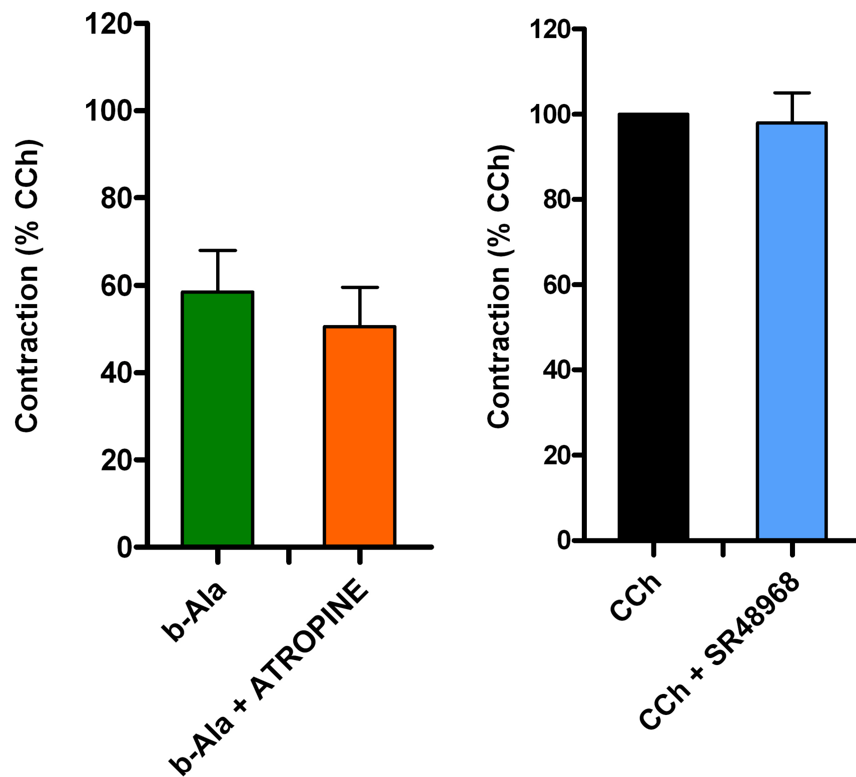


Fig. 5- Histogram showing the effects of the NK2 receptor agonist, β-Ala (1 μM) in the absence or in the presence of the muscarinic receptor antagonist, atropine (1 μM, n = 5), and of CCh (10 μM) in the absence or in the presence of the NK2 receptor antagonist, SR48968 (0.1 μM, n = 5) in human colon. Data are means ±SEM and are expressed as percentage of the maximal effect induced by 10 μM CCh. *P < 0.05 when compared to the respective own control condition.

Discussion

Ang II, the main effector peptide in the renin–Ang system (RAS), is known to elicit a wide range of diverse cellular responses, including growth, proliferation and vascular smooth muscle contraction. It has gradually become evident that in addition to the 'circulating RAS', there is also a 'local RAS' able to generate all bioactive Ang peptides in several tissues and organs, making RAS also a paracrine–autocrine system (Paul et al. 2006, Fyhrquist & Saijonmaa 2008). Ang II acts via cell surface receptors subdivided into AT1 or AT2 type, characterized using selective ligands (De Gasparo et al. 2000). The AT1 receptor mediates all classical actions of Ang II in cardiovascular, renal, neuronal, endocrine, hepatic and other target cells. These actions contribute to the maintenance of arterial blood pressure, electrolyte and water balance, thirst, renal function and structural remodelling of cardiovascular tissue (De Gasparo et al. 2000, Jackson 2001). In rodents, two isoforms of the AT1 receptor are expressed, termed AT1A and AT1B (Johren et al. 2003). AT2 receptors, on the other hand, have been suggested to counterbalance most effects that Ang II exerts through the AT1 receptors (Volpe et al. 2003). The presence of Ang II receptors at various levels along the GI tract has been demonstrated in human, guinea-pig and rat (Wang et al. 2005, Ewert et al. 2006, Spak et al. 2008), suggesting a potential physiological action. Studies of immunohistochemical, in human colon, demonstrated the presence of both Ang II receptor AT1Rs localized in vessel walls, myofibroblasts, and macrophages in lamina propria, crypt bases, and surface epithelium while the AT2Rs found in mesenchymal cells and weakly in parts of surface epithelium (Hirasawa K et. al., 2002).

Another, AT2 receptors are expressed at certain locations in the adult organisms such as in the adrenal gland, brain and myocardium as well as in the vasculature (Fyhrquist & Saijonmaa 2008). Data from our experiments indicate that in mouse and human colon, a local RAS system exists supporting a local action played by Ang II (and its receptors) in the regulation of the gastrointestinal function. The modulation of the colonic contractility and the consequent impact on intestinal transit can be one of the physiological mechanisms by which Ang II would control body fluid and electrolyte homoeostasis. In particular, Ang II positively modulates the spontaneous contractile activity of mouse and human colon via activation of post-junctional and pre-junctional AT1receptors (AT1A receptors in mice), the latter located on the enteric nerves modulating the release of excitatory neurotransmitters.

Ang II in mouse colon

Data from our experiments indicate that in the longitudinal muscle of mouse colon Ang II induced a concentration-dependent contractile effect in in both proximal and distal segments. The concentration–response curve was significantly shifted to the right by the AT1 receptor antagonist losartan, indicating an AT1 receptor mediated effect. The calculated pA2 values are in line with other reports (Schambye et al. 1994, De Godoy & De Oliveira 2002). Moreover, Ang II effects were unaffected by the AT2 receptor antagonist, PD123319. These observations are in agreement with the previous studies in the human oesophageal muscle (Casselbrant et al. 2007), in the guinea-pig, rat and human small intestine (Hawcock & Barnes 1993, Ewert et al. 2006, Spak et al. 2008) and in the guinea-pig stomach (Lu et al. 2011) where AT1 receptors are known to mediate mainly Ang II excitatory effects. Due to the results obtained by RT-PCR, it appears that the AT1

receptors subserving contractile effects belong to the subclass of AT1A receptors. It has been suggested that AT2 receptor activation counterbalances most effects that Ang II exerts through the AT1 receptors (Nouet & Nahmias 2000, De Godoy & De Oliveira 2002, Rattan et al. 2002, De Godoy et al. 2003) via the release of inhibitory autacoids (Siragy & Carey 1999, Israel et al. 2000). In our preparations, we never observed inhibitory/ relaxant effects of Ang II, although tissues were able to relax in response of isoproterenol. Because Ang II binds to its two receptor subtypes, AT1 and AT2, with a similar affinity, the tissue response is highly dependent on the relative responsiveness of both receptors (Nouet & Nahmias 2000). Therefore, when the AT1 subtype is inhibited and the AT2 receptor is free to interact with Ang II, the AT2-mediated effect becomes predominant (De Godoy & De Oliveira 2002, Rattan et al. 2002, De Godoy et al. 2003). In our preparation, the AT2 receptor antagonist, PD123319, did not affect Ang II-induced contraction even when the AT1 subtype receptors are inhibited by losartan. Results from PCR analysis showed that in control condition, there is not a detectable expression of AT2 receptors in the proximal colon, whilst a low expression is evident in the distal colon. However, it should also be noted that AT2 receptor expression may vary according to tissue conditions, such as hypoxia or inflammation (Volpe et al. 2003, Smith & Missailidis 2004). Further studies should address whether conditions may exist in which AT2 receptor expressions are increased in mouse colon and whether this is associated with an altered response to Ang II. Tetrodotoxin partially antagonized the Ang II-induced responses in the proximal colon response suggesting that, in this region, AT1A receptors are localized both at pre-junctional level, where would act modulating neurotransmitter release from the enteric nerves, and at post-junctional level, likely on the smooth muscle cells.

Indeed, TTX abolished the effects of Ang II in distal colon, suggesting a prevalent localization of AT1A receptors at pre-junctional level in this region. Pre-junctional AT1 receptors, mediating Ang II effects, have been demonstrated in the guinea pig small intestine (Hawcock & Barnes 1993, Wang et al. 2005), whilst Ang II acts primarily through AT1 receptor located on the musculature in the isolated human and rat small intestine (Ewert et al. 2006, Spak et al. 2008) and human oesophagus (Casselbrant et al. 2007). In our preparations, the contractile response mediated by pre-junctional AT1A receptor activation was due to the involvement of cholinergic and tachykinergic pathways, because it was antagonized by atropine and by the selective antagonist of NK1 receptor, SR140333. Either atropine or SR140333 reduces Ang II responses to a level not significantly different from that measured in the presence of TTX. Moreover, the observation that, when atropine and SR140333 were applied in combination, there were not any additive effects indicates that acetylcholine and tachykinins, likely substance P, are subsequently involved in the mediation of the indirect responses to Ang II in mouse colon. In particular, we may suggest that Ang II would induce release of substance P by enteric nerves, which acting on NK1 receptors, in turn, would induce release of acetylcholine, being the final contractile mediator, because, as already shown (Mule' et al. 2007, Matsumoto et al. 2009), the contraction induced by a selective NK1 receptor agonist was reduced by atropine, whilst SR140333 did not affect carbachol-induced muscular contraction. This conclusion differs from what observed in guinea-pig small intestine where Ang II responses are due to activation of angiotensin receptors located neuronally on both cholinergic and tachykinergic nerves (Hawcock & Barnes 1993). Specie and tissue differences may account for this discrepancy. Moreover, because 5-HT through 5-HT_3 receptor activation is involved in the regulation of intestinal contractility via modulation of

neurotransmitter release either from cholinergic or noncholinergic neurones (Tuladhar et al. 2000, Chetty et al. 2006, Denes et al. 2009), we tested the possibility 5-HT_3 receptor activation could be involved in Ang II-induced contraction. Such a hypothesis can be discarded because Ang II effects were not modified by ondasetron, 5-HT_3 receptor antagonists. Indeed, in the neural circuit activated by Ang II, are not involved cholinergic interneurones as result by the lack of efficacy of hexamethonium treatment. In conclusion, the presence in the murine colon of the components of RAS suggests that Ang II is also locally generated to control mouse colon motility. In particular, Ang II positively modulates the spontaneous contractile activity via activation of post-junctional and pre-junctional AT1A receptors, the latter located on the enteric nerves and modulating the release of tachykinins and acetylcholine. Tachykinergic neurones and cholinergic neurones are sequentially recruited by Ang II to induce muscular contraction.

Ang II in human colon

Our experiments indicate that in human circular muscle of sigma colon Ang II induced, as in mouse colon, a concentration-dependent muscle contraction. These results are in agreement with studies previously performed in human different gastrointestinal (Fishlock and Gunn, 1970; Ewert et al. 2006b; Ludtke et al. 1989; Casselbrant et al. 2007; Spak et al. 2008). Ang II contractile effects were inhibited by losartan, AT1 receptor antagonist, and insensitive to the treatment with AT2 receptor antagonist, PD123319. Once more, we did not observe any effects due to activation of AT2 receptors even when the AT1 subtype receptors were blocked by losartan. Moreover, our data suggest that in the colon AT1 receptors are localized both at post-junctional level, likely on the smooth muscle cells and at pre-junctional level on enteric neurons, since TTX partially antagonized

the Ang II-induced responses. The observation that in our preparations the contractile response mediated by pre-junctional AT1 receptor activation was due to the involvement of tachykinergic NK2 pathways, because it was antagonized by the selective antagonist of NK2 receptor, SR48968, at a level not significantly different from that measured in the presence of TTX. The observation that atropine was ineffective on Ang II-induced contraction ruled out an involvement of cholinergic motor pathways in the Ang II contractile effects, as previously reported by Fishlock and Gunn (1970). Moreover the contraction induced by by a selective NK2 receptor agonist was not affected by atropine or TTX, indicating that NK2 receptor–mediated smooth muscle contraction of the human colon is a result of direct action on smooth muscle. On the other hands, immunohistochemical analysis demonstrated NK2 receptor immunoreactivity in the smooth muscle layers of human colon (Nakamura et al., 2011). Therefore, it is possible to conclude that in human sigma colon pre-junctional AT1 receptor activation would induce release of tachykinins, likely NKA, from enteric nerves, which acting on NK2 receptors would cause muscular contractile effects. Results from PCR analysis showed that transcripts encoding for AT1 and AT2 receptor subtypes and for angiotensin-converting enzyme (ACE) were expressed in the whole thickness colonic preparations, lacking mRNA expression for angiotensinogen (AGT) and for renin. Actually, Hirasawa et al. (2002) detected all the components of RAS in human colon mucosa, therefore the lack of mRNA expression for AGT and renin in our whole thickness muscular samples may be not indicative of a non functioning local RAS. In conclusion, in human sigmoid colon Ang II positively modulates the spontaneous contractile activity via activation of post-junctional and pre-junctional AT1 receptors, the latter located on the enteric nerves and modulating the release of tachykinins, which in turn, via activation of NK2 receptors, would contribute to the contractile effects.

Conclusions

Overall, our experiments indicate that in mouse and human colon, a local RAS system exists supporting a local action played by Ang II (and its receptors) in the regulation of the gastrointestinal function. Major species differences between human and animal colonic muscle are revealed by our study. In rodents, Ang II contractile effects are primarily due to release of substance P by enteric nerves, which acting on NK1 receptors, in turn would induce release of acetylcholine, being the final contractile mediator. Indeed, in circular muscle from human sigmoid colon, we found that responses to Ang II were insensitive to atropine, being antagonized only by NK2 receptor antagonists. This observation strengthened, also, the conclusions suggesting that tachykinins are the main excitatory neurotransmitters in human sigmoid colon and that NK2 receptors are the major contributor to tachykinin-induced smooth muscle contraction in the human colon (Cao et al., 2000, 2006). Moreover, Ang II was able to affect intestinal contractility at a dose similar to the plasma concentration (about 0.01 nM) detected under basal conditions (Cholewa & Mattson 2005; Jensen et al., 2013) only in mouse colon indicating that in this specie Ang II receptors can be targeted by Ang II formed both at distance (endocrine action) and locally (paracrine action) regulating colonic motility even in physiological condition. In human, higher plasma concentrations of Ang II can be reached under different conditions i.e. during fluid deprivation and thus be responsible of the thirst-induced colonic motility changes (decreased stool frequency and stool weigh). Moreover, Ang II may be locally produced by intestinal RAS increasing its level over the circulating concentration and thus regulating colonic motility. Anyway, the modulation of the colonic contractility and the consequent impact on intestinal transit can be one of the physiological mechanisms by which Ang II would control body fluid and electrolyte

homoeostasis. Moreover, evidence suggests that RAS dysfunction may potentiate immune-based diseases such as IBD, raising the possibility that local RAS system may become a potential therapeutic target in various gastrointestinal diseases (Garg et al. 2012). Lastly, we are aware that, in addition to the key mediator octapeptide Ang II, other AGT fragments have been shown to be biologically active, such as angiotensin 1–7 and Ang III and IV, and this will deserve attention. In conclusion, the presence in the murine and human colon of the components of RAS suggests that Ang II can be also locally generated to control colon motility. In particular, Ang II positively modulates the spontaneous contractile activity of mouse and human colon via activation of post-junctional and pre-junctional AT1 receptors (AT1A receptors in mice), the latter located on the enteric nerves modulating the release of excitatory neurotransmitters.

Capitolo 4

Arginine vasopressin, via activation of post-juntional V1 receptors, induces contractile effects in mouse distal colon

Rationale and aims

Vasopressin (Vp), a critical regulator of water homeostasis by controlling the insertion of aquaporin2 (AQP2) onto the apical membrane of the renal collecting duct (Nielsen et al. 1993, 1995, Fushimi et al.1997), has been reported to regulate electrolyte and water transport in the colon both in vivo and in vitro (Dennhardt R et. al, 1979; Levitan R et. al, 1968; Bridges RJ et. al, 1983 and 1984; Knobloch SF et. al, 1989; Mobasheri A et. al, 2005; Vincentini-Paulino 1992). As already reported, the multiple actions of AVP are mediated by members of G-protein–coupled receptor family divided into three subtypes: V1 receptors (known as V1a), V2 receptors and V3 receptors (previously known as V1b). V1a and V1b receptors effectively couple to G_q and phospholipase C pathway, V2 receptors couple to G_s and adenylyl cyclase pathway (Koshimizu TA et. al, 2012). Moreover, V1 receptors via phospholipase A_2/cyclooxygenase pathway may induces prostaglandin E_2 production in different organs and tissues (Fleisher-Berkovich S, et. al, 2004; Nakatani Y, et. al, 2007). The presence of AVP receptor mRNA at various levels along the GI tract has been demonstrated in humans (Monstein et al 2007) and V1a receptors has been found on the neurons of myenteric plexus in rat stomach, where AVP has been suggested to act as neuromodulator of enteric cholinergic neurons inducing excitatory effects on the contractility (Junfang Qin et. al, 2009). Unclear and conflicting are the studies about a possible effect of AVP in the control of gastrointestinal motility.

Voderholzer WA et al. (1995) suggested that AVP at physiological level might not influence colonic motility in rats and humans while Ward et al. (1997) reported that AVP at low concentration increased slow wave activity and phasic contraction in the muscle strips from dog colon, whilst at higher concentration it caused an inhibitory effect. Recently AVP was shown to inhibit the contractions through production of nitric oxide in strips of the circular muscle of rat colon (Jing H, et. al, 2011). These data would suggest that AVP may be one of the brain–gut peptides influencing gastrointestinal functions also acting at peripheral level. Thus, taken into account that the role of AVP in the bowel motility is far from being clear the aim of this study was to analyze pharmacologically the effects of AVP on mouse distal colon motility, to characterize the subtype(s) of receptor(s) involved and to investigate the action mechanism.

Results:

Circular and longitudinal muscle strips of mouse distal colon displayed spontaneous activity consisting of phasic contractions with amplitude of 238.8 ± 0.5 mg and 244.2 ± 0.8 mg (n = 20), a frequency of 4.2 ± 1.5 c.p.m. and 5.2 ± 1.5 c.p.m (n = 20), respectively. AVP (0.001 nM–100 nM) was without any effects on the circular muscle, whilst it caused concentration-dependent contractile effects on the longitudinal muscle (EC50 = 0.05 nM; 95% CL 0.03–0.1 nM, n = 10) (Figs. 1, 2).

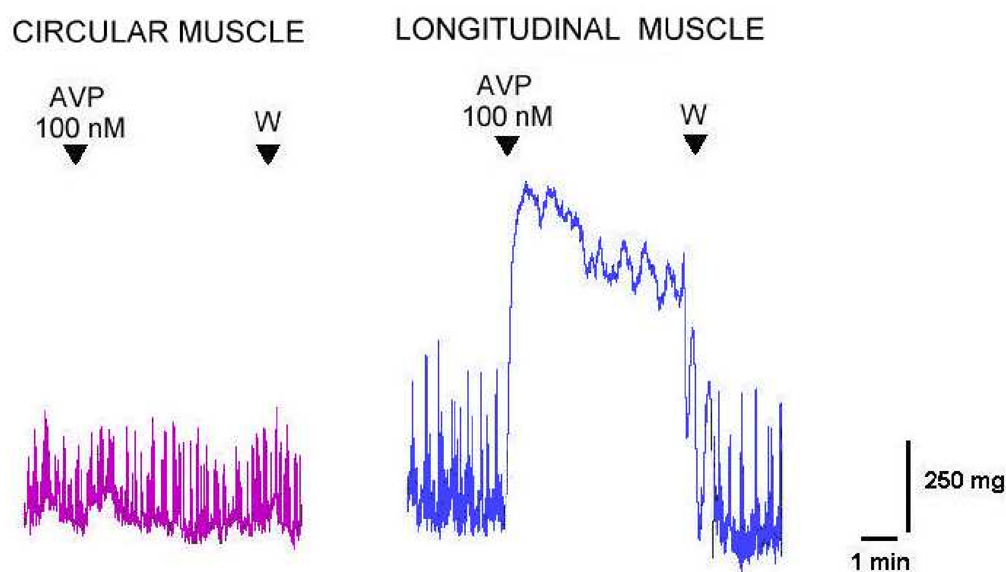
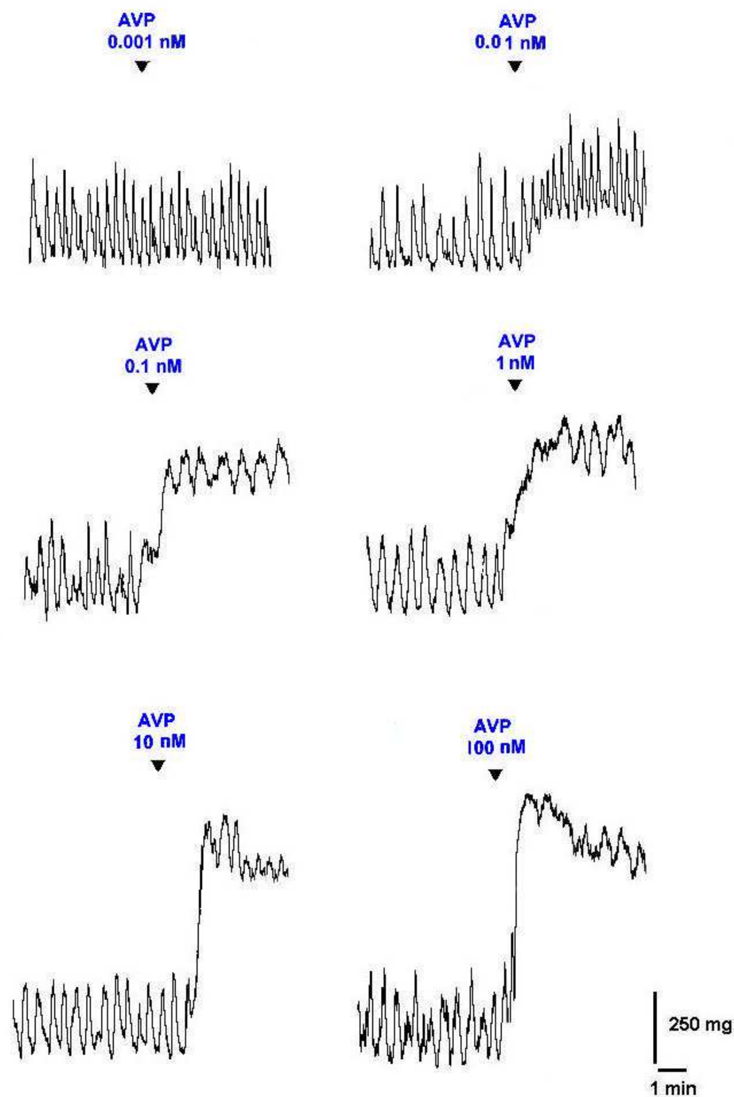


Fig. 1- AVP induced a contractile response only in the longitudinal muscle of mouse distal colon. Original recordings showing the effects evoked by AVP in circular and longitudinal of mouse distal colon.

These effects lasted throughout the drug application time. The maximal response was observed at the dose of 100 nM consisting in an absolute increase in muscular tone of 580 ± 7.6 mg (n = 10). We never observed an inhibitory effect in response to AVP, even at higher concentrations.

The possible involvement of V1 receptor subtype in the response to AVP was tested using, a potent V1 receptor antagonist, V-1880. V-1880 (30 nM), which per se did not modify spontaneous activity (the amplitude and frequency of the spontaneous contraction was 249.1 ± 0.9 mg and 5.0 ± 1.2 c.p.m. in the presence of V-1880, n = 10), shifted to the right the concentration–response curve to AVP (EC50 = 0.9 nM; 95% CL 0.6–1 nM, n = 10) (Fig. 2).

(A)



(B)

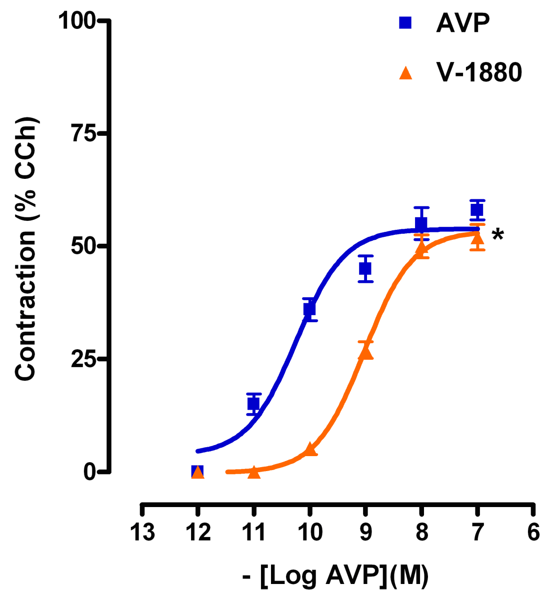


Fig. 2- AVP induced a contractile response in the longitudinal muscle of mouse distal colon via activation of V1 receptors. A) Original recordings showing the dose-dependent effects evoked by AVP in the longitudinal of mouse distal colon. B) Concentration–response curves to AVP before and after V-1880 (30 nM), V1 receptor antagonist, in the longitudinal muscle of mouse distal colon. Data are means ± SEM (n = 10) and are expressed as percentage of the maximal effect induced by 10 µM CCh. *P b 0.05 versus control.

AVP-induced effect was not modified in the presence of a Na^+ voltage-gated neural channel blocker, TTX (1 µM), or atropine (1 µM), a muscarinic receptor blocker, or indomethacin (10 µM), a cyclooxygenase inhibitor (Fig. 3).

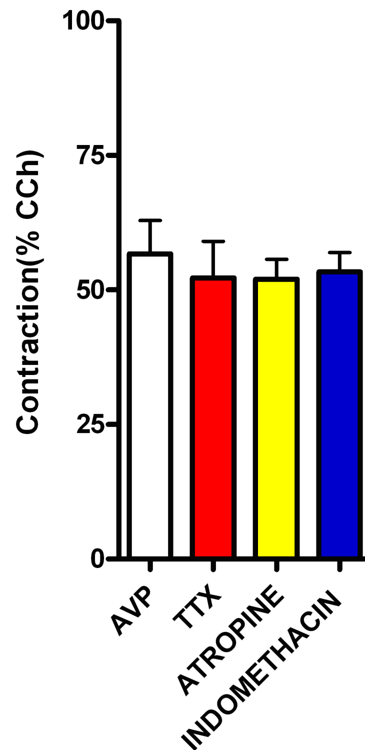


Fig. 3- AVP contractile responses are independent by enteric nerve activation and prostaglandin synthesis. Histogram showing the effects of AVP (10 nM) in the absence or in the presence of TTX (1 μM), Na+ voltage-gated neural channel blocker, atropine (1 μM),muscarinic receptor antagonist, or indomethacin (10 μM), cyclooxygenase inhibitor, in the longitudinal muscle of mouse distal colon. Data are means ± SEM (n = 5 each) and are expressed as percentage of the maximal effect induced by 10 μM CCh. The graphed value for the control bar is the mean of the control data obtained before each treatment.

None of these agents had any effect on the spontaneous mechanical activity. The amplitude and frequency of the spontaneous contraction was: 209.1 ± 1.2 mg and 4.8 ± 1.4 c.p.m. in the presence of TTX (n = 5); 191.3 ± 2.2 mg and 5.3 ± 0.9 c.p.m. in the presence of atropine (n=5); or 234.8±0.9 mg and 5.2 ± 1.3 c.p.m. in the presence of indomethacin (n = 5). In Ca^{2+}-free solution or in the presence of an L-type calcium channel blocker, nifedipine (10 nM), the contractile response to the submaximal dose of AVP (10 nM) was reduced to about 30% of the control (Fig. 4).

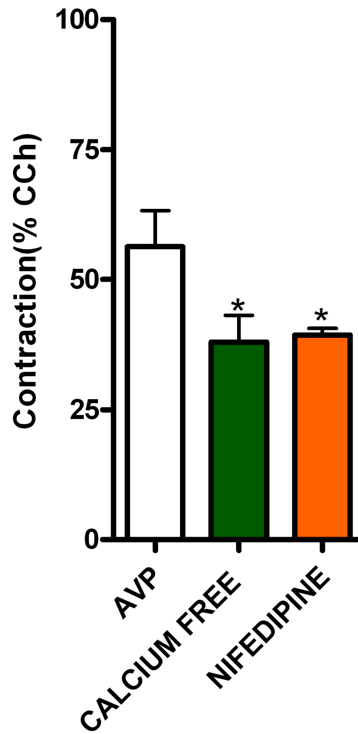


Fig. 4- AVP contractile responses require Ca^{2+} influx from L-type channel. Histogram showing the effects of AVP (10 nM) in longitudinal muscle of mouse distal colon in control condition, in Ca^{2+}-free solution or in the presence of nifedipine (10 nM), L-type calcium channel blocker, in the longitudinal lmuscle of mouse distal colon. Data are means ± SEM (n = 5 each) and are expressed as percentage of the maximal effect induced by 10 μM CCh. The graphed value for the control bar is the mean of the control data obtained before each treatment. *P b 0.05when compared to the respective own control condition

The amplitude and frequency of the spontaneous contraction was 81.1 ± 0.9 mg and 1.2 ± 0.6 c.p.m. in Ca^{2+}-free solution (n = 5); 83.2 ± 0.7 mg and 1.6 ± 0.7 c.p.m. in the presence of nifedipine (n = 5). Contractile response to AVP was, also, tested after depletion of calcium intracellular stores. Depletion of calcium intracellular stores was induced by repetitive challenges with carbachol (10 μM), cholinergic agonist, which mobilized calcium from intracellular stores, in calcium-free medium and in the presence of CPA (10 μM), to block reuptake of Ca^{2+} by sarcoplasmic

reticulum-specific Ca^{2+}-ATPase. AVP was tested once CCh had no effect on the colonic muscle. In this condition, the contractile response to AVP (10 nM) was abolished, indicating mobilization of calcium from intracellular stores after activation of V1 receptors (Fig. 5).

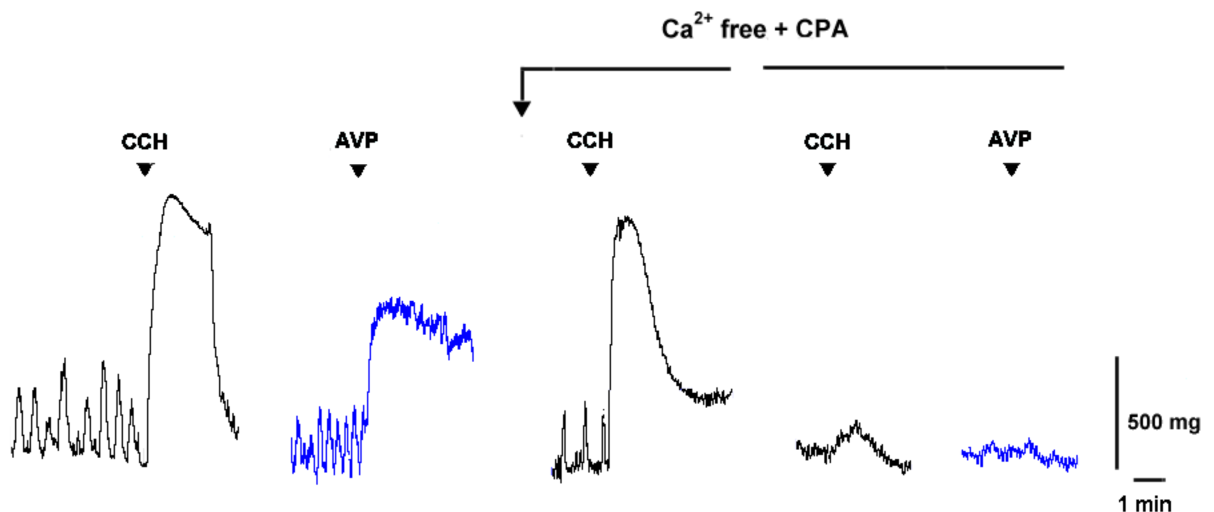


Fig. 5- AVP contractile responses require calcium release from intracellular stores. Original tracings showing the effects of AVP (10 nM) on spontaneous mechanical activity of longitudinal muscle of mouse distal colon before and after depletion of intracellular Ca^{2+} stores achieved by repetitive addition of carbachol (10 μM) in Ca^{2+}-free solution and in the presence of CPA (10 μM). Note that in this condition, the contractile response to AVP was abolished.

Since intracellular Ca^{2+} release from the sarcoplasmic reticulum is accomplished in the gastrointestinal muscle by activation of intracellular signaling transduction, the excitatory effect induced of submaximal dose of AVP was tested in the presence of U-73122, an inhibitor of the PLC, and in the presence of DDA, an adenylyl cyclase inhibitor. U-73122 (50 μM) antagonized the response by 70%, whilst, as expected, DDA (10 μM) was without any effect (Fig. 6).

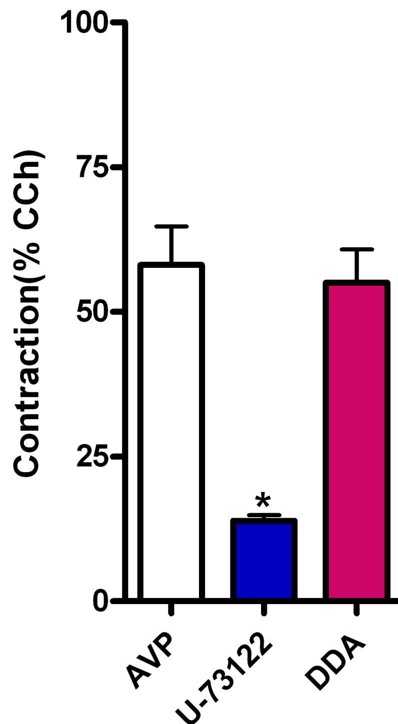


Fig. 6- Phospholipase C is activated by V1 receptors. Histogram showing the effects of AVP (10 nM), in the absence or in the presence of U-73122 (50 μM), an inhibitor of the phospholipase C, or DDA (10 μM), an adenylyl cyclase inhibitor, in the longitudinal muscle of mouse distal colon. Data are means ± SEM (n = 5 each) and are expressed as percentage of the maximal effect induced by 10 μM CCh. The graphed value for the control bar is the mean of the control data obtained before each treatment. *P b 0.05 when compared to the respective own control condition.

Lastly, we tested the effects of oxytocin, which differs from AVP by only two amino acids, since it is reported to modulate intestinal motility in rats (Qin J et. al, 2009). In our preparation, oxytocin induced an excitatory effect in the longitudinal muscle of distal colon only at very high concentrations, from 0.1 μM. However, this effect was antagonized by V-1880 (30 nM), indicating an aspecific binding of oxytocin to V1 receptors (data not shown).

Discussion

The results of this study show that AVP, via activation of V1 receptors, is able to modulate positively the contractile activity of longitudinal muscle of mouse distal colon, independently by enteric nerve activation and prostaglandin synthesis. Contractile response is achieved by increase in cytoplasmatic Ca^{2+} concentration via extracellular Ca^{2+} influx from L-type Ca^{2+} channels and via Ca^{2+} release from intracellular stores through PLC pathway. No modulation has been observed on the contractility of the circular muscle. The role of AVP in the regulation of free water reabsorption, body fluid osmolality, blood volume, blood pressure, cell contraction, cell proliferation, and adrenocorticotropin secretion, is well know, but different experimental evidences suggest an involvement of this hormone also in the control of GI functions (Schapiro H et. al, 1972). In humans, intravenous administration of AVP, used in the treatment of upper GI haemorrhages, causes abdominal cramps and a strong urge to defecate, likely by stimulating colonic motility (Zhu YR et. al, 1992). Either central and peripheral site of action have been reported (Bueno L, et al, 1992). Indeed AVP can be produced locally in cells of human and rat gastrointestinal system (Friedmann AS et. al, 1991) and AVP receptors are reported to be expressed throughout gastrointestinal tract (Monstein HJ et. al, 2008; Qin J et. al, 2009). However, there are contradictory reports of the effects of vasopressin being different the responses in the various parts of the GI tract and among species. In our experiments, AVP induces a concentration-dependent contractile response on the longitudinal muscle of mouse distal colon by activation of specific V1 receptors, as shown by the sensitivity of the response to the selective V1 receptor antagonist V-1880.

Actually, local V1 receptors mediating either AVP excitatory or inhibitory effects have been reported in other animal preparations (Ward SM et. al, 1997; Qin J et. al, 2009; Jing H et. al, 2011). In our preparations, we never observed inhibitory effects of AVP even at the higher concentrations, although tissues were able to relax in response of isoprotenerol. Moreover, AVP responses can be selectively seen only in longitudinal muscle strips, being AVP ineffective, at our concentration range, on the circular muscle.
It has been reported that in mouse gastrointestinal muscle there is a differential control of the two muscular layers, being the longitudinal muscle layer under a more prominent excitatory control than the circular muscle layer (Daniel EE, et. al, 2004). Thus the effects of an excitatory drug only on the longitudinal muscle cannot be considered as an exception. Moreover, the contractile effects induced by AVP were insensitive to drugs blocking neural conduction and cholinergic neurotransmission, indicating a recruitment of post-junctional V1 receptors. This conclusion is in agreement to results obtained in dog proximal colon (Ward SM et. al, 1997), but it is in contrast with other studies in monkey and rats (Qin J et. al, 2009; Jing H 2011; Zhu YR et. al, 1992), which suggest neural mechanisms underlying AVP effects, strengthened species differences in the response to AVP. Furtherly, our study demonstrated that contractile effects of AVP are dependent upon increase in intracellular Ca^{2+} concentration due to Ca^{2+} influx from extracellular space *via* L- type Ca^{2+} channels nifedipine-sensitive and Ca^{2+} release from internal stores. Moreover, we showed that contractile responses to AVP were reduced in the presence of the PLC inhibitor, U-73122, indicating that, also in our preparation, V1 receptors are coupled to the Gq protein/PLC_{β} pathway leading to the increase in intracellular calcium concentration by release from intracellular stores. In addition, AVP, *via* activation of V1 receptors/phospholipase A_2/cyclooxygenase, has been shown to stimulate prostaglandin E2 production human fibroblasts

(Nakatani Y, et. al, 2007). However, in our preparation, prostaglandin synthesis seems not to be involved in the excitatory effects to AVP due to the insensitivity of the contractile response to indomethacin. As expected, AVP responses were resistant to adenylyl cyclase inhibitor and this observation may allow us also to exclude an involvement of V2 receptors, generally linked to adenylyl cyclase activation and increase of intracellular cAMP. Physiological plasma levels of AVP in rat and in mouse were comparable to those in human, being in the order of 10-11 M (Oliverio MI et. al, 2000; Yang Y et. al, 2002). In our experiments, AVP at such concentrations marginally influences colonic motility, as reported in rat colon (Voderholzer WA et. al, 1995). However, higher plasma concentrations of AVP can be reached after water deprivation at the early stage of septic shock (Wilson MF et. al, 1981; Sharshar T et. al, 2003; Jochberger S et. al, 2006) in various chronic inflammatory states (Girón-González JA et. al, 2004; Han DM et. al, 2007), and during the treatment of critical illness (Singer M. 2008; Russell JA et. al, 2008). In addition, expression of AVP-like immunoreactivity was found in the GI wall, increasing in oral to aboral direction (Friedmann AS et. al, 1993; Voderholzer WA et. al, 1995, Zhu YR et. al 1992; Vasallo JL et. al, 1992), suggesting a possible local source of AVP that increasing AVP level over the circulating concentration may be one of the factors regulating colonic motility. Therefore, as suggested for Ang II (Mastropaolo M, et. al, 2013), the modulation of the colonic contractility and the consequent impact on intestinal transit can be one of the mechanisms by which AVP would control body fluid and electrolyte homeostasis. Lastly, AVP differs from oxytocin by only two amino acids. Oxytocin has been reported to induce in rats excitatory effects on the gastric motility (Qin J et. al, 2009), but not on the colonic motility (Ataka K et. al, 2012).

In our experiments, we could see an excitatory effect to oxytocin only at very high concentration (over 0.1 μM). This effect was antagonized in presence of the V1 receptors antagonist, V-1880, suggesting that, at such high concentrations, oxytocin interacts aspecifically with V1 receptors. Thus, similarly from what has been reported in rat colon (Ataka K et. al, 2012), in mouse colon oxytocin seems not to play a role in the modulation of contractility.

Conclusions

In conclusion, in this study we reported that AVP can induce muscular contraction in longitudinal distal mouse by activation of V1 excitatory receptors located at the post-junctional level. V1 receptors determine increase in cytoplasmatic Ca^{2+} concentration via extracellular Ca^{2+} influx from L-type Ca^{2+} channels and via Ca^{2+} release from intracellular stores through PLC pathway. Overall our results underlie the possibility that AVP is able to modulate intestinal motility also acting at peripheral level, although its physiological significance remains to be elucidated.

Capitolo 5

General Discussion

The small and large intestine play a key role in maintenance of body fluid homeostasis by regulating the transport of ions by the intestinal epithelium. A large volume of fluid enters the small intestine daily from ingestion and from secretions of the gastrointestinal tract, but only about 0.1-0.5 liter is eliminated in the feces. It is known that absorption of nutrients, water and electrolytes in the intestine can be affected by alteration of motility. The precise coordination of epithelial function and smooth muscle activity necessary to maintain normal gut function or to respond to injurious challenges is a prime function of the enteric nervous system. It is unique in its ability to function independently of the central nervous system in the control of the functions of the digestive tract. For this reason, the ENS is considered to be a "second brain in the gut" (Gershon MD, 1999). However, the central nervous system is able to modulate the GI function by sending instructions via the two components of the extrinsic autonomic nervous system. Hormones and paracrine mediators released by different source cells may directly influences central neuronal function and thereby gut motility. However for many of them the presence of specific receptors at different levels in the ENS, smooth muscle cells or other effectors (i.e. ICC) has been demonstrated, indicating a peripheral site of action (Hansen MB, 2003). Ang II and AVP have been demonstrated to be able to modulate the processes of absorption/secretion of ions and water in the intestine, thus, it has been suggested their involvement in the modulation of the intestinal contractility as part of the physiological mechanisms controlling body fluid and electrolyte homoeostasis.

Moreover, restriction of water intake has been reported to decrease stool frequency and stool weight in volunteers (Klauser et al., 1990). These effects of thirst could be mediated by an action of systematically released hormones on colonic smooth muscle. Moreover, evidence suggests that RAS dysfunction may potentiate immune-based diseases such as inflammatory bowel disease (Garg et al., 2012) and vasopressin *via* V1 receptors seem to have proinflammatory properties in experimental-induced colitis (Ferrier et al., 2010), raising the possibility that these hormonal systems may become a potential therapeutic target in a various gastrointestinal diseases. However, despite these encouraging observations, several aspects pertaining to the regulation of digestive functions by Ang II and AVP remain unclear and deserve extensive investigation. Considering that the presence of receptors for Ang II and AVP at various levels along the GI tract in human, guinea pig, and rat (Wang et al. 2005, Ewert et al. 2006, Spak et al. 2008), we addressed the possibility that Ang II and AVP might be involved in the regulation of intestinal (colon) motility acting at peripheral level, using an approach *in vitro* in which the influence of external factors is removed, but the intestinal muscle itself performs in a manner analogous to its *in vivo* capacity. Both mouse and human colonic muscle segments/strips have been used since results obtained in experimental animals cannot be immediately translated to the humans, due to species differences. Moreover, molecular analysis allowed us to verify expression of receptor subtypes and of the other components of the hormonal systems considered. The results presented in this thesis show that two hormones, Ang II and vasopressin, involved in the control of body fluid and electrolyte homeostasis, are able to influence intestinal motility activating specific receptors located both in the intestinal smooth muscle effectors and in the enteric nerves. Interestingly, both hormones induce similar effects both in mice and in humans. In fact, as we

showed Ang II via activation of AT1 positively modulates spontaneous activity in both preparations.

However, in circular muscle from human sigmoid colon, we found that responses to Ang II were insensitive to atropine, being affects only by NK2 receptor antagonists. Therefore, our studies may strengthen the conclusions suggesting that tachykinins are the main excitatory neurotransmitters in human sigmoid colon (Cao et al., 2000, 2006), stressing the existence of specie differences and indicating caution in translate data from animals to humans. It as to be underlined the presence in the murine and human colon of components of RAS, which suggests that angiotensin receptors can be targeted by Ang II formed both at distance (endocrine action) and locally (paracrine action) to control colonic motility.

AVP induce muscular contraction in longitudinal distal mouse, not in the circular one, by activation of V1 excitatory receptors located at the post-junctional level. the excitatory effects induced on the longitudinal muscle may contribute to determine the overall muscular tone instead of propulsive motor activity. Tonic activity would allow homogenizing of the luminal contents and increasing both the pressure and the area of their contact with the mucosal surface, facilitating water and salt absorption.

Preliminary experiments seem to indicate that AVP, at high concentrations, is able to contract as well the human colon (Fig.1).

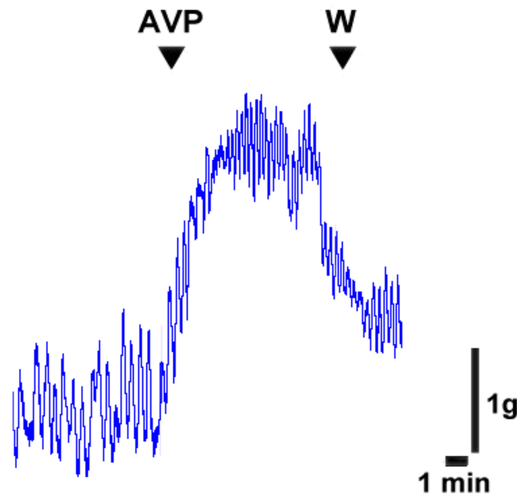


Fig.1- Original recordings showing the mechanical responses evoked AVP in human colon.

These concentrations are far from systemic AVP concentrations, although remains the possibility of local source of AVP that increasing AVP level over the circulating concentration may contribute to the regulation of the colonic motility. Thus, further experiments are needed to fully elucidate the role played by AVP on colonic motility in human under physiological/pathological conditions. In conclusion, results present in this thesis indicate that the modulation of the colonic contractility and the consequent impact on intestinal transit can be one of the physiological mechanisms by which Ang II and AVP would control body fluid and electrolyte homoeostasis.

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CURRICULUM

Studies reported in this thesis are part of a broad research project aimed to investigate the role played in the control of GI motility under physiological conditions by different molecular systems, which are supposed to be involved at different levels also in the development, maintenance and/or prevention of some widespread GI diseases as the inflammatory bowel diseases. Results obtained have been published in international journals, subjected to peer-review and quoted on JCR-ISI database.

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- Mastropaolo M, Zizzo M.G., Belluardo N, Serio RM "Defects in dopaminergic pathway affect contractility of colon from hypoxanthine-guanine phosphoribosyltransferase (HPRT) knockout mice. "In: VII Congresso annuale del Dipartimento di biologia cellulare e dello sviluppo Palermo dicembre 2009.

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- Auteri M., M.G. Zizzo, M. Mastropaolo, R. Serio. “Gaba & “little brain”: ruolo emergente nel controllo della motilità intestinale”. Meeting Ibim-Cnr Biotecnologie: ricerca di base, interdisciplinare e traslazionale in ambito biomedico 27-28 giugno 2013.

- Zizzo MG.; Mastropaolo M; Auteri M ;Mule’ F.; Serio R. “Postnatal development of the 5-hydroxytryptamine (5-ht) signaling system in the mouse duodenum”. 64° Congresso Nazionale Società italiana Fisiologia (SIF) 18-20 Settembre 2013.

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- Mastropaolo M; Zizzo MG; Mulè F; Serio R. “Angiotensin II positively modulates the spontaneous contractile activity of mouse and human colon via activation of at1 receptors”. Ngm 2012 - joint international neurogastroenterology and motility meeting, bologna 6-8 settembre 2012.

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- Zizzo MG; Mastropaolo M; Cavallaio G; Mosca F; Serio R. “Postnatal developmental changes in enteric dopaminergic system”. The pediatric academic societies (PAS) annual meeting, washington, 4-5 maggio 2013.