Universitat Autònoma de Barcelona

NEUROMUSCULAR TRANSMISSION IN THE GASTROINTESTINAL TRACT AND ITS INTERACTION WITH PACEMAKERS

NOEMÍ MAÑÉ REED

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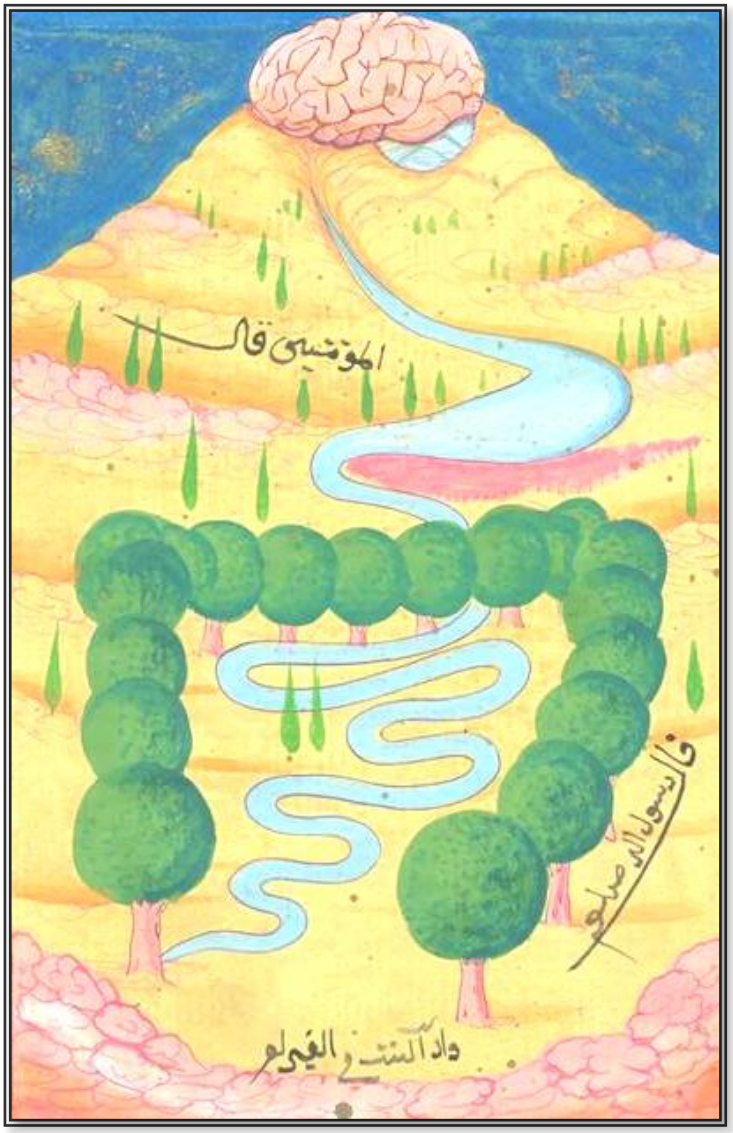

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NEUROSCIENCE DOCTORAL PROGRAM

FACULTY OF VETERINARY MEDICINE

DEPARTMENT OF CELLULAR BIOLOGY, IMMUNOLOGY AND PHYSIOLOGY

UNIVERSITAT AUTÒNOMA DE BARCELONA

DISSERTION ADVISOR: MARCEL JIMÉNEZ FARRERONS

Universitat Autònoma de Barcelona

Neuromuscular transmission in the gastrointestinal tract and its interaction with pacemakers

By

Noemí Mañé Reed

A dissertation submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

Neuroscience Doctoral Program

Department of Cell Biology, Physiology and Immunology

Universitat Autònoma de Barcelona

Advisor

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FACULTAT DE VETERINÀRIA

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Bellaterra, Barcelona

2016

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I certify that the dissertation entitled "Neuromuscular transmission in the gastrointestinal tract and its interaction with pacemakers" submitted by Noemí Mañé Reed in partial fulfillment of the requirements for the Degree of Doctor of Philosophy was carried out under my supervision and I authorize the submission to undertake its oral defense.

In witness whereof, I hereby sign this document,

Professor titular d'Universitat a la Unitat de Fisiologia, Departament de Biologia Cel·lular, Fisiologia i Immunologia; Facultat de Veterinària; Universitat Autònoma de Barcelona

Faig constar que la tesi titulada " Estudi de la relació neuromuscular al tracte gastrointestinal i la seva interacció amb l'activitat marcapassos" presentada per Noemí Mañé Reed per optar al grau de Doctora ha estat realitzada sota la meva supervisió i autoritzo la presentació per realitzar la seva defensa oral.

I, perquè així consti, signo aquest document,

Dr. Marcel Jiménez Farrerons,

Doctoral advisor/ Director de tesi

This work has been funded by the Ministerio de Ciencia e Innovación (BFU2009-11118) and by the Agència de Gestió d'Ajuts Universitaris de Catalunya (2011 CTP 00032).

Noemí Mañé has been funded by the Ministerio de Ciencia e Innovación (AP2012-0897)

Acknowledgements

First of all, I would like to acknowledge my PhD supervisor Marcel Jiménez. Without your guidance this work would have not been possible. You are an outstanding professor, supervisor and most important of all, a great person.

Thank you to all my predecessors Víctor, Diana and Míriam, for having the patience to show me everything I know. I want to thank my colleagues Marina, Mònica, Javier, Sergio B, Sergio L, Roberto, Eva, Joan, Ferran, Sandra, Asun and Elena for their fabulous companionship. To Ricardo, Alberto, Margalida, Vero and Andrea.

Thanks to the technical support and kindness of Antonio, the unique Emma and Claudia. I would also like to thank Maite, Patri, Esther and Vicente for their support during my stay at the Unit.

I want to thank my friends from "Gluten Free", "Tronkas", "floretes quedem demà?", "Croquetes" and "Alcohol na mas" for the always amazing times spent together during these years, so necessary to keep on going. My flat mates and dog walking friends.

Thanks to my amazing family, both Mañés and Reeds, specially my mum, my dad and my sister. Regardless of the distance, they are always with me. To my dogs Avi, Jerry and Rua and to my mouse Priscilla.

It may sound strange, but I would like to dedicate my thesis to the animals I've used for my studies. I wish I could return them what I hope they have contributed to science.

Last but not least, thanks to Marina, always by my side, for her unconditional love.

Acronyms

ACh acetilcholine

ADP adenosine diphosphate

ADPβS adenosine 5´-β-2-tiodiphosphate

ANO-1 anoctamin-1 channel

ATP adenosine 5'-triphosphate

AUC area under curve

BPTU 1-(2-(2-(*tert*-butyl)phenoxy)pyridin-3-yl)-3-(4-(trifluoromethoxy)phenyl)urea

βNAD beta-nicotinamide adenine dinucleotide

cAMP cyclic adenosine monophosphate

cGMP cyclic guanosine monophosphate

cKIT tyrosine protein-kinase kit

CPI-17 Protein phosphatase 1 regulatory subunit 14A

cpm cycles per minute

CO carbon monoxide

DAG diacylglycerol

DMP deep muscular plexus

EFS electrical field stimulation

ENS enteric nervous system

GC guanylyl cyclase

GPCR G-protein coupled receptor

GTP guanosine triphosphate

HAPC high amplitude propagating contractions

H_2S sulphide

IC_{50} concentration decreasing the original response a 50%

ICC interstitial cells of Cajal

ICC-DMP interstitial cells of Cajal of the deep muscular plexus

ICC-IM intramuscular interstitial cells of Cajal

ICC-MY myenteric interstitial cells of Cajal

ICC-SEP septal interstitial cells of Cajal

ICC-SM submuscular interstitial cells of Cajal

IJP inhibitory junction potential

IJPf fast inhibitory junction potential

IJPs slow inhibitory junction potential

IP_3 inositol 1,4,5-triphosphate

IPAN intrinsic primary afferent neurons

L-NNA Nω-nitro-L-arginine

MLC myosin light chain

MLCK myosin light chain kinase

MLCP myosin light chain phosphatise

MP myenteric plexus

MRS 2179 N6-metil 2'-deoxiadenosine 3',5'-bisphosphate

MRS 2279 (1*R**,2*S**)-4-[2-Chloro-6-(methylamino)-9*H*-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester

MRS 2500 (1*R**,2*S**)-4-[2-Iodo-6-(methylamino)-9*H*-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester

NANC non adrenergic non cholinergic

NaNP sodium nitroprusside

nNOS neuronal nitric oxide synthase

NO nitric oxide

NSCC non-selective cation channels

ODQ 1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one

PACAP pituitary adenylate cyclase activating polipeptide

PDGFRα platelet derived growth factor receptor alfa

PIP_2 phosphatidylinositol 4,5-bisphosphate

PKC protein kinase C

PKG protein kinase G

PLC phospholipase C

RMP resting membrane potential

$SK_{(Ca)}$ small conductance calcium activated potassium channels

SMC smooth muscle cell

SMP submuscular plexus

SP substance P

TTX tetrodotoxine

VIP vasoactive intestinal polypeptide

TABLE OF CONTENTS

ABSTRACT

Neuromuscular transmission in the gastrointestinal tract and its interaction with pacemakers

The large intestine performs a variety of functions essential for an optimal use of food. Different types of motility patterns are needed to develop these functions and, in this thesis, our aim has been to increase the understanding of the mechanisms underlying their regulation.

The temporal and spatial coordination of smooth muscle contractions and relaxations results in the different motor patterns. Smooth muscle is under the control of two systems: the Enteric Nervous System and the Interstitial Cells of Cajal (ICC) networks.

The major transmitter substances released by enteric inhibitory motor neurons are ATP and nitric oxide (NO). In this work, we have described how different neuronal firing frequencies are responsible for the action of one or another co-transmitter. Short bursts or low frequencies cause mainly $P2Y_1$ mediated purinergic responses (blocked by BPTU, a novel $P2Y_1$ allosteric antagonist) while long bursts at high frequencies enhance nitrergic neurotransmission. This is due to the fact that purinergic neurotransmission attenuates with frequency-increase and therefore, this pathway is only able to cause transient relaxations. Nitrergic neurotransmission, in contrast, can relax colonic tissue in a sustained manner. Accordingly, nitrergic responses are predominant in the proximal colon where storage of ileal effluents and absorption of water and other substances take place. Contrarily, the gradient of purinergic neurotransmission is inverse and therefore, transient relaxations predominate in the distal colon.

Inhibitory neurotransmission can modulate the myogenic rythmicity generated by ICCs. Whereas slow waves, originated in the colonic ICCs of the Submuscular Plexus (ICC-SMP), appear to be resistant to mild hyperpolarisations, cyclic depolarizations developed by the ICC of the Myenteric Plexus (ICC-MY) disappear when the membrane potential goes under -40 mV due to their dependence on L-type calcium channels. Therefore, in order to allow the development of this pacemaker, the resting membrane potential (RMP) in the area were cyclic depolarizations occur is around -40 mV. In contrast, smooth muscle cells near the submuscular plexus have a

RMP of around -50 mV, i.e., a transwall gradient exists. In consequence, the amplitude of inhibitory responses is also graded.

Slow waves can be recorded in vivo as “ripples”: high frequency contractions of relatively low amplitude; while cyclic depolarizations have been associated to propulsive contractions and we believe they are also the myogenic basis of neural induced high amplitude propagating contractions. In vitro, we have observed a third motor pattern that consists in a wax and wane of slow waves associated contractions. The wax and wane is very likely due to a modulation of the ICC-SMP pacemaker by the ICC-MY pacemaker. We believe this pattern is the basis of the single motor contractions observed with high resolution manometry.

In conclusion, the relative contribution of ATP and NO to relaxation depends on the firing frequency of inhibitory motor neurons. They display different functional roles and are distributed along the colon accordingly. Although ICCs settle the rythmicity of contractions through their spontaneous cycling of the RMP, enteric neurons collect information of the luminal content and enhance or inhibit the pacemakers and/or produce relaxations or contractions in the different areas in order to optimize the processing of the content.

Keywords: colon, inhibitory junction potential, co-transmission, nitric oxide, ATP, relaxation, motility

Estudi de la relació neuromuscular al tracte gastrointestinal i la seva interacció amb l'activitat marcapassos

L'intestí gros desenvolupa una varietat de funcions essencials per a una utilització òptima dels aliments. Es necessiten diferents tipus de patrons de motilitat per dur a terme aquestes funcions i, en aquesta tesi, el nostre objectiu ha estat augmentar la comprensió dels mecanismes subjacents a la seva regulació.

La coordinació temporal i espacial de les contraccions i relaxacions del múscul llis donen lloc als diferents patrons motors. El múscul llis està sota el control de dos sistemes: el Sistema Nerviós Entèric i les xarxes de Cèl·lules Intersticials de Cajal (ICC).

Els principals transmissors alliberats per les motoneurones entèriques inhibitòries són l'ATP i l'òxid nítric (NO). En aquest treball, hem descrit com les diferents freqüències de descàrrega de les neurones determinen l'acció d'un o altre co-transmissor. Ràfegues curtes o de baixa freqüència causen principalment respostes purinèrgiques mitjançant l'activació de receptors $P2Y_1$ (bloquejades per BPTU, un nou antagonista al·lostèric del receptor $P2Y_1$), mentre que les ràfegues llargues a altes freqüències potencien la neurotransmissió nitrèrgica. Això es deu a què la neurotransmissió purinèrgica s'atenua amb a altes freqüències i per tant, aquesta via només és capaç de causar relaxacions transitòries. La neurotransmissió nitrèrgica, per contra, pot relaxar el còlon de manera sostinguda. D'acord amb això, les respostes nitrèrgiques són predominants al còlon proximal, on té lloc l'emmagatzematge dels efluents de l'ili i l'absorció d'aigua i altres substàncies. En canvi, el gradient de la neurotransmissió purinèrgica és invers i per tant, al còlon distal, responsable de la propulsió de femtes, hi predominen les relaxacions transitòries.

La neurotransmissió inhibitòria pot modular la ritmicitat miogènica generada per les ICCs. Mentre que les ones lentes, originades per les ICC del plexe submuscular de còlon (ICC-SMP), semblen ser resistents a la hiperpolarització, les despolaritzacions cícliques desenvolupades per les ICCs del plexe mientèric (ICC-MY) desapareixen quan el potencial de membrana baixa per sota de -40 mV a causa de la seva dependència dels canals de calci de tipus L. Per tant, per tal de permetre el desenvolupament d'aquest

marcapàs, el potencial de membrana a la zona on es generen les despolaritzacions cícliques és al voltant de -40 mV. En canvi, les cèl·lules del múscul llis properes al plexe submuscular tenen un potencial de membrana al voltant de -50 mV, és a dir, existeix un gradient transmural. En conseqüència, l'amplitud de les respostes inhibitòries també mostra un gradient.

Les ones lentes són registrades en viu com contraccions d'alta freqüència i d'amplitud relativament baixa ("ripples"); mentre que les despolaritzacions cícliques s'han associat a contraccions de propulsió i creiem que són també la base miogènica de les contraccions d'alta amplitud conegudes com a HAPC induïdes via neural. In vitro, s'ha observat un tercer patró motor que consisteix en un "wax and wane" (créixer i minvar) de les contraccions associades a ones lentes. És molt probable que el "wax and wane" es degui a una modulació del marcapàs del ICC-SMP per part del marcapàs de l'ICC-MY. Creiem que aquest patró és la base dels contraccions individuals ("single motor patterns") observats amb manometria d'alta resolució.

En conclusió, la contribució d'ATP i NO a la relaxació depèn de la freqüència de descàrrega de les motoneurones inhibitòries. Els co-transmissors mostren diferents rols funcionals i estan distribuïts al llarg del còlon en conformitat amb el paper de cada regió. Malgrat les ICCs estableixen la ritmicitat de les contraccions a través dels marcapassos, les neurones entèriques recopilen informació del contingut luminal i potencien o inhibeixen els marcapassos i/o produeixen relaxacions o contraccions en les diferents àrees per tal d'optimitzar el processament del contingut.

Paraules clau: còlon, potencial post-unió inhibitori, co-transmissió, òxid nítric, ATP, relaxació, motilitat

INTRODUCTION

Anatomy and function of the colon

The mammalian colon has evolved to sub-serve its main functions, which are: 1- absorption of water, electrolytes, bile salts, short-chain fatty acids and other bacterial metabolites, 2- storage of colonic contents and 3- propulsion of fecal material (Christensen, 1991). The motility patterns required to accomplish these physiological purposes are complex and variable. Accordingly, motor activity and gross morphology of the colon are adapted so that these processes proceed efficiently (Huizinga & Lammers, 2009).

Regional differences are apparent in the colon as it can be divided into two segments regarding their embryological origin, innervation and blood supply (Christensen, 1991). Morphologically, the ascending or right colon, which derives from the mid-gut, has a saccular appearance. In contrast, the descending or left colon, originated from the hind-gut, has a tubular morphology. These gross morphology characteristics are in accordance with their different physiological role. The right colon acts as a storage site for ileal effluent. To aid maximal absorption of water during this time, the principal motility requirement is slow propulsion, retrograde movements and mixing to favor the contact of the content with the colonic mucosa. Intraluminal contents become progressively more solid as most of the water is absorbed. Colonic motility in the left colon, which acts as a conduit for the passage of feces, must have the ability to mix and propel intraluminal contents towards the rectum. It displays a pattern of intense peristaltic contractions associated with the propulsion of dehydrated feces (Ritchie, 1968;Scott, 2003;Sarna, 1991;Karaus & Wienbeck, 1991;Jameson & Misiewicz, 1993).

Colonic smooth muscle

The effectors of colonic motility are smooth muscle cells (SMCs), which form the *muscularis propria* beneath the submucosa. Layers of SMCs line the walls of various organs and tubes and their contractile function is not under voluntary control. The *muscularis propria* of the colon contains two layers of SMCs oriented perpendicular to each other. The muscle layer adjacent to the submucosa is the circular muscle layer, in which SMCs are circularly aligned and, therefore, responsible for annular contractions. Beneath, between the circular muscle and the serosa, the longitudinal muscle layer contains SMCs displayed perpendicular to circular SMCs (Figure 1).

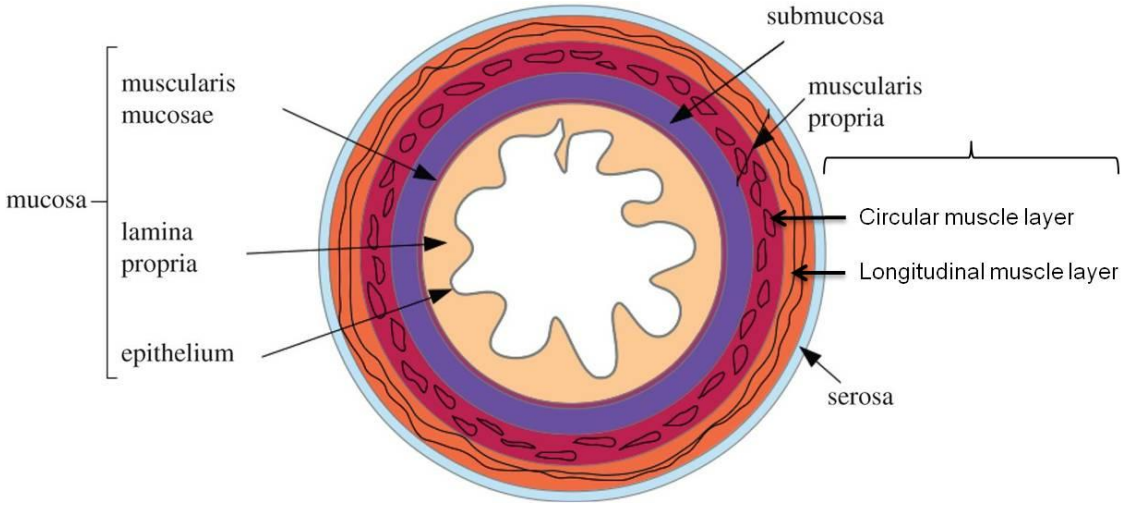

Figure 1. Layers of the gastrointestinal tract. The gastrointestinal tract is composed of concentrically arranged layers. Going from inside the lumen radially outwards the layers are: mucosa, submucosa, circular muscle layer, longitudinal muscle layer and serosa. *Adapted from Balbi & Ciarletta, 2013 (Balbi & Ciarletta, 2013).*

Contraction of SMCs occurs through cross-bridge cycling of myosin with actin, which causes the shortening of the SMC. The contractile activity of SMCs is

determined by the phosphorylation state of the light chain of myosin (MLC), a highly regulated process. For contraction to occur, MLC kinase (MLCK) must phosphorylate the MLC in order to promote the interaction between myosin and actin. Contractile responses are prototypically mediated by G-protein coupled receptors (GPCR) that increase phospholipase C (PLC) activity. PLC produces two second messengers from the membrane lipid phosphatidylinositol 4,5-bisphosphate: diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (Berridge, 1993). IP_3 binds to specific receptors on the sarcoplasmic reticulum and causes the release of calcium (Ca^{2+}) from intracellular stores into the cytoplasm. DAG along with Ca^{2+} activates protein kinase C (PKC), which has contraction-promoting effects through specific target phosphorylation. Cytosolic Ca^{2+} binds to calmodulin, leading to activation of MLCK.

The phosphorylation state of MLC is further regulated by MLC phosphatase (MLCP) which dephosphorylates the MLC leading to relaxation (Barany & Barany, 1980). Phosphorylation of MLCP inhibits its activity leaving the MLC phosphorylated and thereby, maintaining a contractile state (Figure 2).

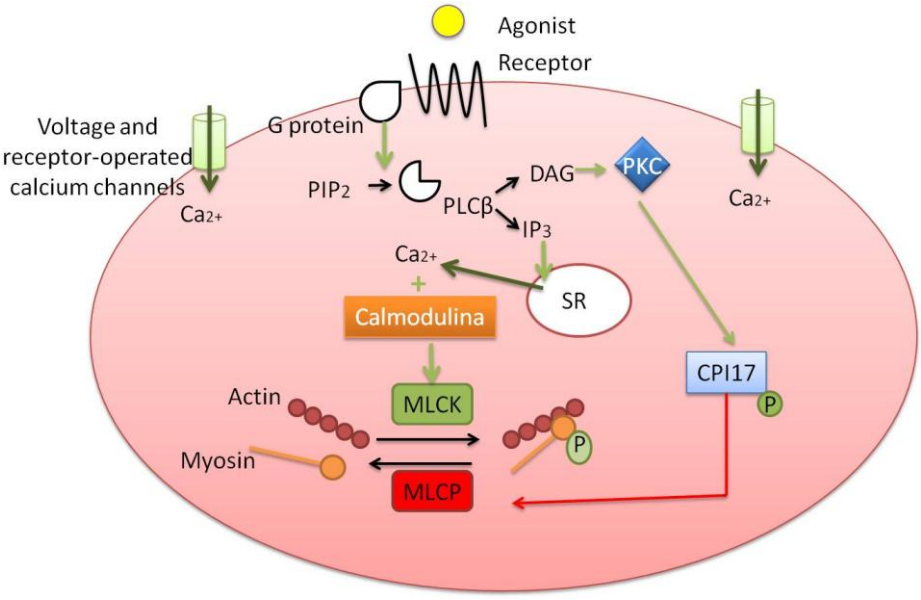

Figure 2. Contractile apparatus of smooth muscle cells. Agonist binding to serpentine receptors activates the G protein which increases phospholipase C (PLC) activity. PLC breaks phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 binds to receptors on the sarcoplasmic reticulum (SR) to release calcium (Ca^{2+}) into the cytoplasm. Ca^{2+} entry through voltage and receptor operated channels also occurs. Cytosolic Ca^{2+} binds to calmodulin to activate myosin light chain kinase (MLCK). MLCK phosphorilates the myosin light chain (MLC) promoting the interaction between actin and myosin. DAG along with Ca^{2+} activates protein kinase C (PKC), which promotes sustained contraction through phosporylation of CPI17, a myosin light chain phosphatase (MLCP) inhibitor.

Relaxation requires a decreased intracellular Ca^{2+} concentration and/or increased MLC phosphatase activity (Morgan & Suematsu, 1990;Somlyo *et al.*, 1999). This state can be achieved by either the removal of the contractile stimulus or by inhibition of the contractile mechanism (Fig. 2). Receptor- and voltage-operated Ca^{2+} channels must be closed to reduce Ca^{2+} entry into the cell (Webb, 2003).

Resting membrane potential (RMP) of SMCs through the gastrointestinal tract varies between -80 and -40 mV. A variety of K^+ channels contribute to setting the RMP of SMC. In most regions, RMP is considerably less negative than the K^+ equilibrium potential (E_K=~−80mV) due to a finely tuned balance between K^+ channels and non-selective cation channels (NSCC) (Koh *et al.*, 1999). Action potentials in colonic SMC result from the activation of voltage-dependent Ca^{2+} channels. Depolarization of the membrane potential enhances the open probability of these channels while

hyperpolarization distances the SMC from the entering of Ca^{2+} and therefore, promotes relaxation.

The level of excitability and contractility of SMCs is regulated by the networks of Interstitial cells of Cajal (ICC) and the more than 100 million neurons that form the enteric nervous system (ENS), which is in turn modulated by the parasympathetic and sympathetic branches of the autonomic nervous system. The findings in the search of the mechanism basis underlying colonic motility reveal strongly preserved elemental features across all mammalian species.

Interstitial cells of Cajal

ICCs were first described in the early twentieth century by Santiago Ramón y Cajal (Cajal, 1893;Cajal, 1892). He first classified them as neurons due to the fact that, together with neurons, ICCs were stained by methylene blue. It was later postulated that the cells identified by Cajal may act as a pacemaker system for the intestinal musculature (Thuneberg, 1982). Ultrastructural studies of ICC and the speculations on the origin of the slow wave activity observed in the RMP of ICC networks and smooth muscle reinforced the theory of ICCs as pacemaker cells in the gut (Faussone Pellegrini *et al.*, 1977;Rumessen & Thuneberg, 1982;Rumessen *et al.*, 1982;Thuneberg, 1982). Later on, studies using mutant mice that lacked ICCs showed an absence of pacemaker activity in the mouse small intestine. Furthermore, other studies were able to record rhythmic electrical activity in isolated ICCs (Koh *et al.*, 1998;Thomsen *et al.*, 1998).

ICCs are of a mesenchymal origin (Young *et al.*, 1996) and can be specifically identified using antibodies against the tyrosine kinase receptor cKIT (Ward *et al.*,

1995;Torihashi *et al.*, 1995;Huizinga *et al.*, 1995). However, the labeling of the anoctamin-1 (ANO-1) channel, a chloride channel, has been proposed as a more specific marker (Gomez-Pinilla *et al.*, 2009) as cKIT is also expressed in mast cells which can invade the tissue in particular situations such as inflammation (Loera-Valencia *et al.*, 2014). Based on their anatomical location in the muscle wall, several subpopulations of ICC have been identified. According to their distribution, the ICCs found in the colon (Alberti *et al.*, 2005) can be classified into (Faussone-Pellegrini *et al.*, 1990) (Figure 3):

- ICCs of the submucosal layer (ICC-SM) (Rumessen *et al.*, 1993): The axis of ICC-SM lies parallel to circular SMCs. They form a loose network through small processes that make contact with neighboring ICC-SM.

- ICCs of the intermuscular layer or myenteric (ICC-MY): ICC-MY lie between the circular and longitudinal muscle layers. They are multipolar and form a three dimensional network through multiple primary processes which contact and connect electrically with neighboring cells (Komuro, 2006).

- ICCs found throughout the circular and longitudinal muscle (ICC-IM): ICC-IM are bipolar and run parallel to the surrounding SMCs.

- ICC in the septal regions (ICC-SEP): ICC-SEP are located between and surrounding muscle bundles of large animals including dogs (Horiguchi *et al.*,

2001;Horiguchi *et al.*, 2003;Ward & Sanders, 1990), monkeys (Blair *et al.*, 2012) and humans (Liu *et al.*, 2012).

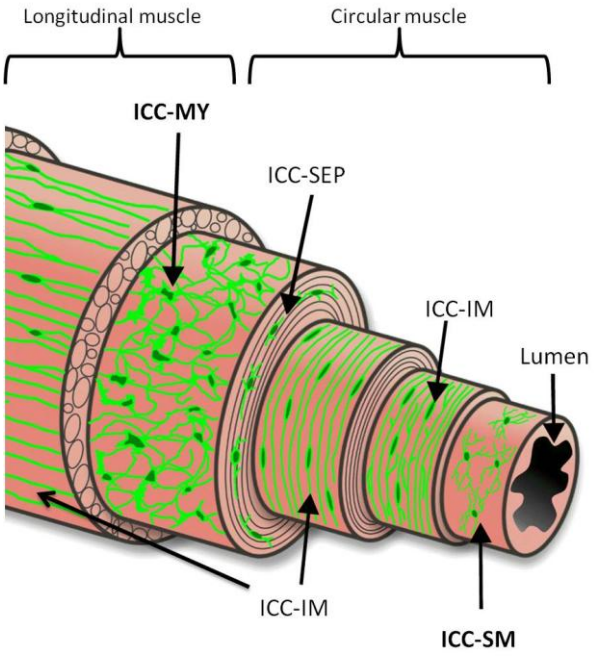

Figure 3. Organization of ICCs through the colonic wall. From the lumen to the exterior, submucous ICCs (ICC-SM) lie beneath the submucosa. Intramuscular ICC (ICC-IM) are located within the circular and longitudinal muscle layers. Myenteric ICCs (ICC-MY) lie between the circular and longitudinal muscle layers. Only large mammalians including dogs, primates and humans have septal ICCs (ICC-SEP) lying in the circular muscle layer. *Adapted from (Blair et al., 2014).*

The slow waves displayed by ICC are cyclic spontaneous transient depolarizations of their membrane potential as a result of spontaneous inward currents. The intracellular mechanism underlying generation of pacemaker activity is not fully understood. It is believed that the release of Ca^{2+} from intracellular stores results in transient activation of ANO1 channels, producing Cl^- outward currents (Huizinga *et al.*,

2002;Hwang *et al.*, 2009). Depolarization of ICC activates voltage-dependent Ca^{2+} channels, generating slow waves. Termination of slow waves occurs by reuptake of Ca^{2+} into the endoplasmic reticulum via the sarco/ER Ca^{2+}-ATPase pump and the entrance of Cl^- through the Na-K-Cl transporter.

ICCs communicate via low resistance connections named gap junctions to coordinate the spread of slow waves (Rumessen *et al.*, 1993;Huizinga *et al.*, 1995). ICCs also form gap junctions with neighboring SMCs (Daniel & Wang, 1999), an essential feature required to convey slow wave activity to SMCs. SMCs respond to pacemaker activity with activation of voltage-dependent Ca^{2+} currents and K^+ currents. These conductances yield depolarization, Ca^{2+} entry, and initiation of contraction (Figure 4).

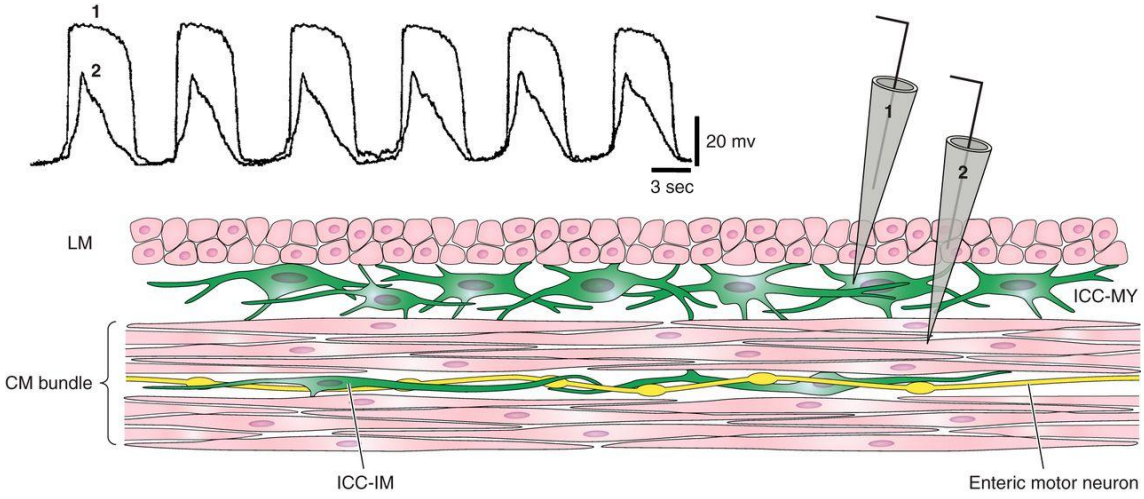

Figure 4. ICCs are interconnected by gap junctions, which also connect ICCs to SMCs and interconnect SMCs. Spontaneous slow waves generated in ICCs (1) are conducted to SMCs (2) (Sanders *et al.*, 2014).

In the colon, slow waves are originated in the ICCs located along the submucosal surface of the circular muscle layer (ICC-SM) (Langton *et al.*, 1989;Pluja *et al.*, 2001). Their frequency is between 2-4 cycles per minute in the human colon (Ford *et al.*, 1995;Latimer *et al.*, 1981;Narducci *et al.*, 1987;Taylor *et al.*, 1975) and between 10 and 15 cycles per minute in rodents. Another pacemaker known as cyclic depolarizations developed most probably by the ICC-MY (Carbone *et al.*, 2013;Pluja *et al.*, 2001) can be recorded. Cyclic depolarizations occur at a frequency of approximately 1 cycle per minute in the rat and mouse colon (Pluja *et al.*, 2001;Costa *et al.*, 2013) and between 0.3-0.6 cycles per minute in the human colon (Rae *et al.*, 1998) in *in vitro* conditions. Both pacemakers are conducted to SMCs which develop contractions at equivalent frequencies if depolarization reaches the opening threshold of L-type calcium channels (above -40 mV) in *in vitro* conditions (Figure 5). The nature of each pacemaker has been shown to be different. The generation of cyclic depolarizations is dependent on L-type calcium channels since it has been shown that they are sensitive to nifedipine, an L-type calcium channel blocker. Slow waves, in contrast, persist under the blockage of L-type calcium channels (Pluja *et al.*, 2001) (Table 1).

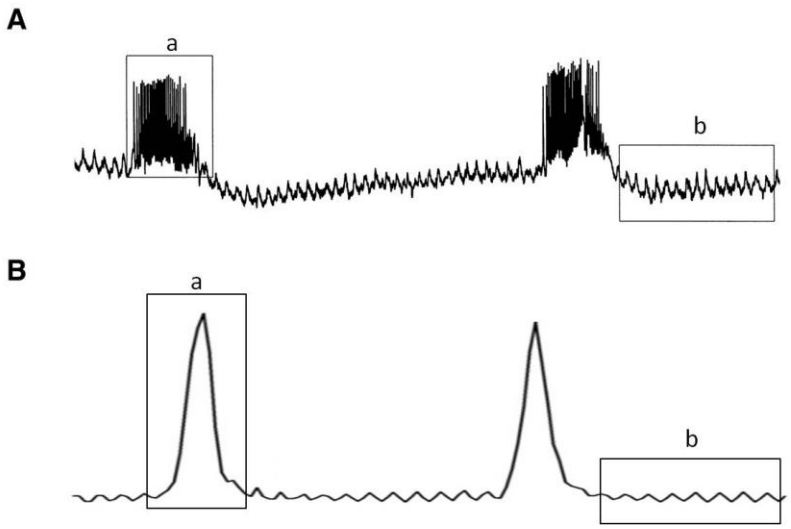

Figure 5. Pacemakers displayed by the ICC-SM and the ICC-MY. The ICC-MY display cyclic depolarizations (Aa) that are the basis of low frequency contractions (Ba). The ICC-SMP displays slow waves (Ab) that are the basis of high frequency contractions (Bb). *Adapted from (Pluja et al., 2001).*

In vitro, both types of contractions appear to be TTX insensitive, showing that they are not originated by enteric neurons. High frequency contractions associated to slow waves are typically seen at the same frequency in vivo and are widely known as ripples (Narducci *et al.*, 1987;Dinning *et al.*, 2014). They are most often recorded in the distal and sigmoid colon and high resolution manometry has permitted to witness that most of them are propagated in a retrograde direction. Their aim is to mix fecal material while enhancing absorption but can also display a propulsive role, contradicting previous data. Retrograde ripples also help contain and control defecation by resisting anally-directed flow (Dinning *et al.*, 2014). Isolated pressure events named single motor patterns are also recorded in vivo with very similar features, such as propagation rate, amplitude and duration, to cyclic myogenic contractions.

Regarding cyclic depolarizations, in contrast, no in vivo motility pattern occurring at the same frequency as *in vitro* low frequency contractions has been recorded. As a consequence, no in vivo motility pattern has been directly linked to cyclic depolarizations.

		Low-frequency contractions		High-frequency contractions	
Human	In vivo (manometry)	**High amplitude propulsive contractions (HAPCs)**	1 per hour (Dinning *et al.*, 2010)	**Rhythmic low amplitude contractions**	2-4 cpm (Taylor *et al.*, 1975;Latimer *et al.*, 1981;Narducci *et al.*, 1987;Ford *et al.*, 1995)
	In vitro (whole colon)	**Colonic motor complexes (CMCs)**	0.25 cpm (Spencer *et al.*, 2012)		
	In vitro (colonic strips)	**Slow long duration contractions**	0.3–0.6 cpm (Rae *et al.*, 1998)	**Slow-wave associated contractions**	2-4 cpm (Rae *et al.*, 1998)
Mouse	In vitro (whole colon)	**Propagating peristaltic contractions** **Colonic migrating motor complexes**	0.9 – 2 cpm (Costa *et al.*, 2013;Mule *et al.*, 1999) 0.3 cpm (Fida *et al.*, 1997;Fida *et al.*, 1997;Powell *et al.*, 2003)	**Ripples**	13-15 cpm (Yoneda *et al.*, 2002)
	In vitro (colonic strips)	**Spontaneous contractions**	0.9 ± 0.1 cpm (Domenech *et al.*, 2011)	*No SMP*	
Rat	In vivo (strain gauge)	**Giant migrating contractions**	0.6 cpm (Li *et al.*, 2002)	**Rythmic phasic contractions**	10-13 cpm (Li *et al.*, 2002)
	In vitro (whole colon)	**Rythmic propulsive motor complexes**	0.3–2 cpm (Huizinga *et al.*, 2011)	**Ripples**	11.3 ± 0.8 cpm (Huizinga *et al.*, 2011;Costa *et al.*, 2013)
	In vitro (colonic strips)	**Low frequency high amplitude contractions**	0.5–1.5 cpm (Pluja *et al.*, 2001)	**High frequency low amplitude contractions**	13–15 cycles/min (Pluja *et al.*, 2001)

Table 1. Low and high frequency contractions recorded using different methodologies in the human, murine and rat colon.

After a meal, retrograde cyclic motor patterns increase and a motor pattern named high-amplitude propagating contractions (HAPC) appears. The high amplitude of these contractions allows them to transport high amounts of fecal material for long distances (Dinning *et al.*, 2014) (Figure 6).

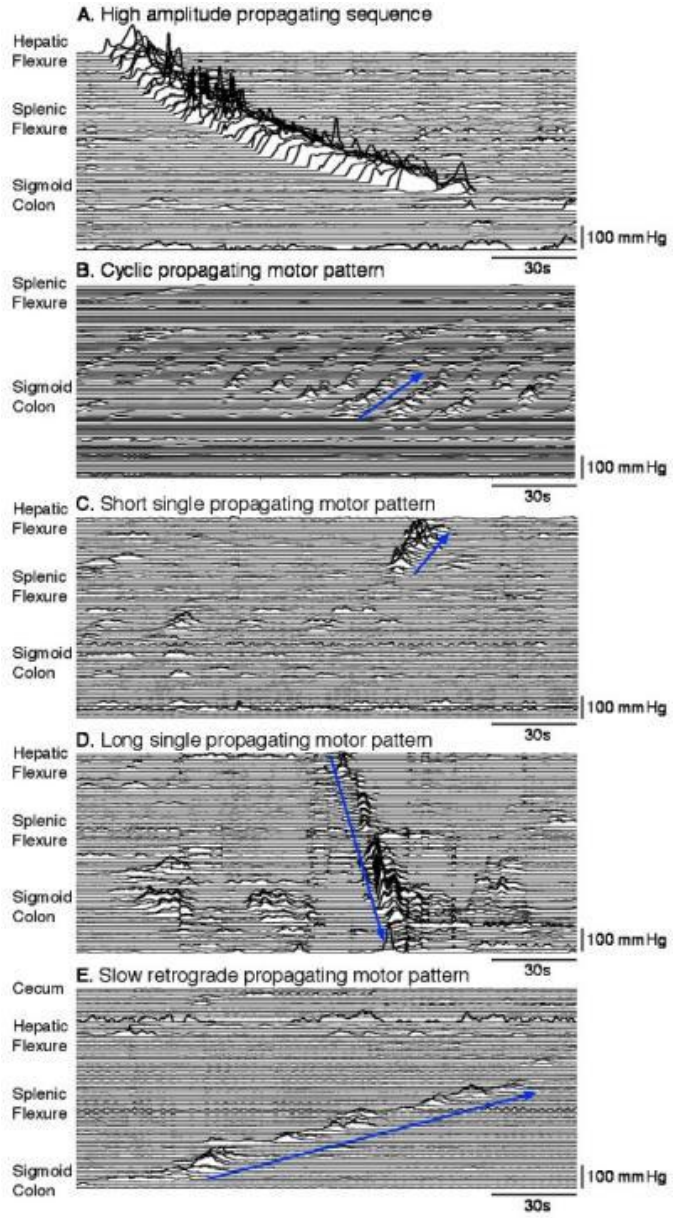

Figure 6. Main types of propagating motor patterns observed with high resolution manometry. (A) High-amplitude propagating sequence; (B) cyclic retrograde propagating motor pattern; (C) short single retrograde propagating motor pattern; (D) long single propagating motor pattern and (E) Slow retrograde propagating motor pattern (Dinning *et al.*, 2014).

Enteric nervous system

Enteric neural control is essential for an optimal mixing and propulsion of luminal contents in the gastrointestinal tract (Kunze & Furness, 1999). The ENS is organized in a myenteric and submucous plexus (Figure 7). The submucosal plexus or Meissner's plexus located within the submucosa layer contains neurons that innervate the mucosa and its blood vessels and is involved in regulating the functions of the mucosa. The myenteric plexus or Auerbach's plexus, aligned between the circular and longitudinal layer, mainly regulates muscle activity modulating locally the mixing and propulsive movements of the large intestine (Olsson & Holmgren, 2001;Kunze & Furness, 1999;Bertrand, 2003). According to their functional characteristics, ENS neurons can be classified into afferent neurons, interneurons and motor neurons (Kunze & Furness, 1999;Furness, 2000;Costa *et al.*, 2000;Brookes, 2001).

Afferent neurons can be classified into two groups: intrinsic primary afferent neurons (IPANs) which have their cell bodies in both the myenteric and submucosal ganglia and project their terminals to the intestinal mucosa, and intestinofugal afferent neurons which's neuronal bodies are located in the myenteric plexus ganglia

and their axons migrate from the gut wall to contact the prevertebral sympathetic ganglia (celiac, inferior and superior mesenteric).

Interneurons form chains of neurons that project in oral and aboral direction. They can therefore be classified into upstream and downstream interneurons and have a crucial role in local reflexes.

Motor neurons represent the final motor connection with SMCs of the circular and longitudinal layers. They can be classified into excitatory and inhibitory motor neurons according to the encoded neurotransmitters (Furness, 2000). Excitatory motor neurons mainly encode for acetylcholine (ACh) and tachykinins (mainly neurokinin A and substance P). The proposed as major inhibitory neurotransmitters encoded by motor neurons are NO (Bult *et al.*, 1990) and adenosine 5'-triphosphate (ATP) (Burnstock *et al.*, 1970). Other neurotransmitters such as vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP) and carbon monoxide (CO) have been proposed as inhibitory neurotransmitters but little functional evidence has been provided (see for review (Lecci *et al.*, 2002)).

Within motor neurons there is a small subgroup, secretomotor and vasomotor neurons, which project to the mucosa and local blood vessels.

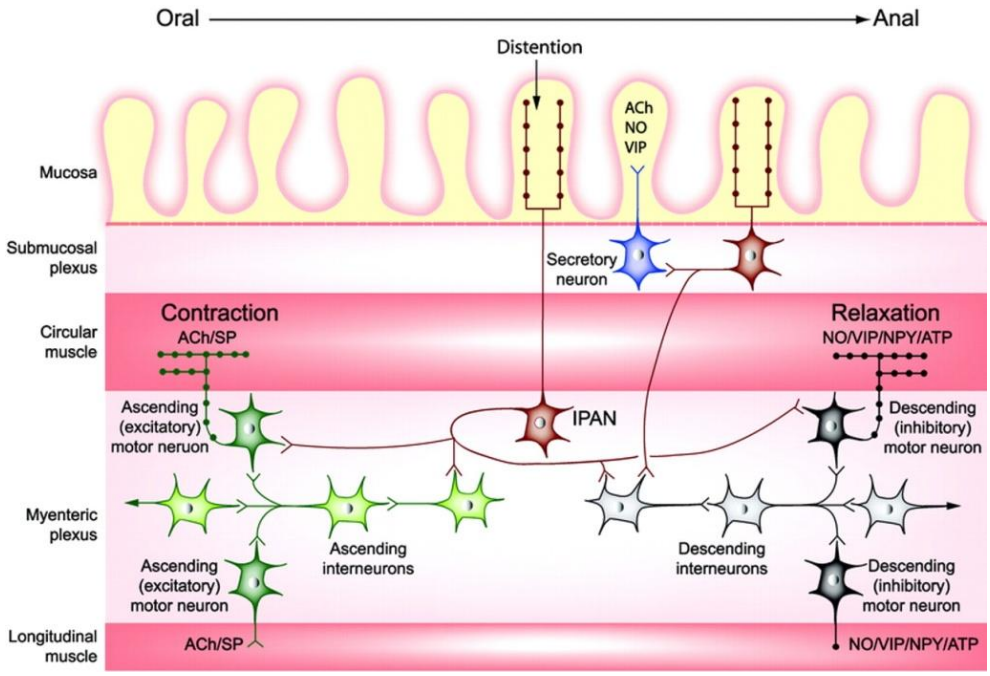

Figure 7. Types of neurons in the intestine: ascending interneurons in green, descending interneurons in grey, IPANs in brown, excitatory motor neurons in dark green, inhibitory motorneurons in dark grey and a secretory neuron in blue. Obtained from Benarroch et al., 2007 (Benarroch, 2007).

The existence of polarized enteric neural pathways (Bayliss & Starling, 1899) allows the propulsion of bolus through the digestive tub due to an oral active contraction and an aboral active relaxation of the circular smooth muscle. For a long period, this mechanism known as ‘peristaltic reflex’ has been considered the basis of the propulsion of luminal contents.

Enteric inhibitory neurotransmission

As mentioned above, some of the ENS motor neurons release inhibitory neurotransmitters. The microelectrode technique allows the measuring of the membrane

potential of individual SMCs of the gastrointestinal tract, using an intracellular electrode (or microelectrode) and an extracellular reference electrode. In this technique, inhibitory motor neurons can be stimulated and a hyperpolarization of the RMP of SMCs can be observed in response to neurotransmitters release. These hyperpolarizations are referred to as inhibitory junction potentials (IJP) (Figure 8). IJP in response to electrical stimulation of intrinsic nerves were first observed in gastrointestinal smooth muscle at the beginning of 1963 (Burnstock *et al.*, 1963). These IJPs were classed pharmacologically as non-adrenergic and non-cholinergic (NANC) (Burnstock *et al.*, 1964;Burnstock *et al.*, 1966), the main neurotransmission pathways of the gastrointestinal tract described until that moment.

IJP in the colon classically display a fast and prominent hyperpolarization followed by a slow and more sustained hyperpolarization that are known as fast IJP (IJPf) and slow IJP (IJPs) respectively (Figure 8) (Crist *et al.*, 1992;He & Goyal, 1993;Gallego *et al.*, 2008a). The human sigmoid colon displays only a IJPf in response to single pulses (Gallego *et al.*, 2008a).

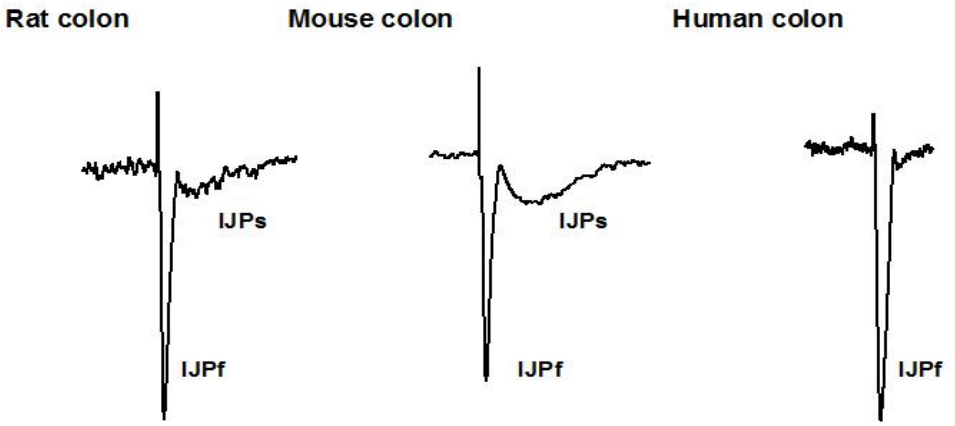

Figure 8. Microelectrode recordings of an IJP produced through stimulation of inhibitory motor neurons in the rat, mouse and human colon. Rodent IJP display both IJPf and IJPs while the human colon only exhibits a IJPf. Notice the presence of stimulus artifacts above the membrane potential recording.

Purinergic neurotransmission

In order to identify a substance as a neurotransmitter, several criteria need to be satisfied:

- The neurotransmitter and the enzymes necessary for its formation must be present in the neuron,
- The neurotransmitter must be released from the neuron when activated,
- The putative neurotransmitter added exogenously must have the same effect as the endogenous neurotransmitter on smooth muscle,

- An enzyme that inactivates the action of the neurotransmitter must be present in the tissue,
- A receptor and a post-junctional pathway that respond specifically to the neurotransmitter must be present,
- Drugs that modify neuron-muscle interaction should also alter the effects of the neurotransmitter when applied exogenously.

Professor Burnstock proved in the early 70s that ATP or a related purine fulfilled all of these criteria as the neurotransmitter released by NANC neurons responsible for inhibitory neurotransmission in the gastrointestinal tract (Burnstock *et al.*, 1970).

Purines exert their action on SMCs through purine receptors. Purine receptors are classified into two families: adenosine receptors (P1) and ATP/ADP sensitive (P2) receptors (Burnstock *et al.*, 1978). P2-purinoreceptors include two groups: P2X and P2Y. P2X receptors are ligand-gated ion channels, while P2Y are GPCR and 8 subtypes are recognized (Alexander *et al.*, 2008;Burnstock, 2009). $P2Y_1$ receptor has been identified as the purine receptor responsible for inhibitory neuromuscular transmission in the gastrointestinal tract (Gallego *et al.*, 2006a). $P2Y_1$ receptors are expressed in the human gut (Janssens *et al.*, 1996), the muscularis externa of rat gut (Van *et al.*, 2007), as well as both the muscularis externa and myenteric plexus in the murine gut (Giaroni *et al.*, 2002;Zhang *et al.*, 2010). Three antagonists of $P2Y_1$ receptors (MRS2500 > MRS2279 > MRS2179 (Grasa *et al.*, 2009)) have been used to characterize the inhibitory pathway in the human colon (Gallego *et al.*, 2006b;Gallego *et al.*, 2008b;Gallego *et al.*, 2011) or small intestine (Gallego *et al.*, 2014) and from

animal tissues such as the pig or guinea pig small intestine (Wang *et al.*, 2007;Gallego *et al.*, 2008c), rat colon (Grasa *et al.*, 2009) and rat and mouse internal anal sphincter (McDonnell *et al.*, 2008;Opazo *et al.*, 2011;Duffy *et al.*, 2012) blocking the fast component of the IJP. Besides the pharmacological evidence of $P2Y_1$ involvement in the fast IJP, an absence of purinergic responses has been observed in $P2Y_1^{-/-}$knockout mice (Gallego *et al.*, 2012;Hwang *et al.*, 2012;Gil *et al.*, 2013) reinforcing the role of the $P2Y_1$ receptor in relaxation of the gastrointestinal tract.

A new $P2Y_1$ antagonist, 1-(2-(2-(tert-butyl)phenoxy)pyridin-3-yl)-3-(4-(trifluoromethoxy)phenyl)urea (BPTU), has been recently discovered by Bristol-Myers Squibb (Chao *et al.*, 2013). The previous $P2Y_1$ antagonists are of a nucleotidic nature and bind to the same site as the endogenous ligand (orthosteric). BPTU, in contrast, binds on the external receptor interface with the lipid bilayer becoming the first allosteric antagonist of a GPCR (Zhang *et al.*, 2015) (Figure 9).

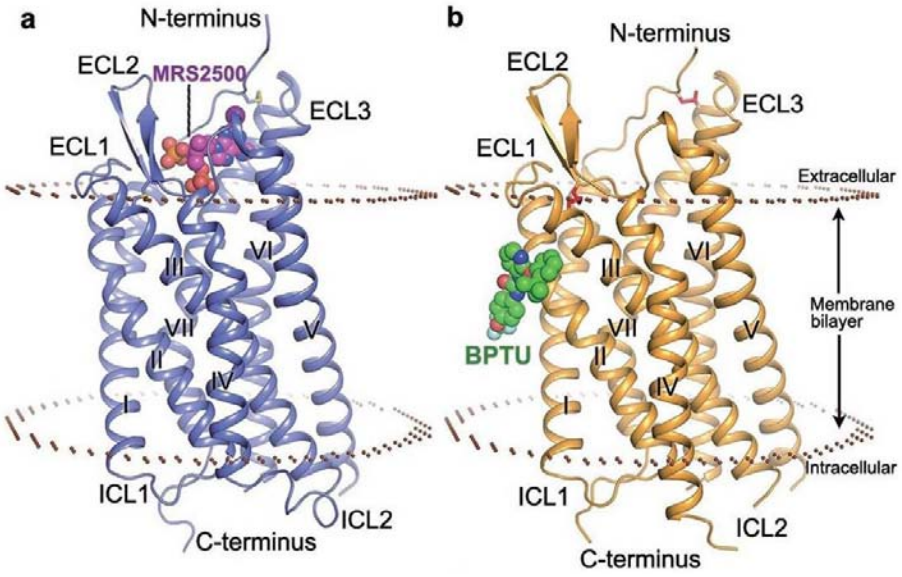

Figure 9. Ligand-binding sites of MRS2500 and BPTU to the $P2Y_1$ receptor. MRS2500 has a nucleotidic nature and binds to the same site as the endogenous purinergic neurotransmitter. The binding site of BPTU, in contrast, is located outside the helical bundle. Obtained from Zhang et al. 2015 (Zhang *et al.*, 2015).

Most of P2Y receptors are coupled to a Gq protein (von, I, 2006). Their activation results in PIP_2 hydrolysis into IP_3 and DAG. IP_3 produces an intracellular calcium mobilization (Gao *et al.*, 2006;Hu *et al.*, 2003) while DAG activates PKC. Cytosolic calcium increase leads to the opening of apamin sensitive small conductance calcium activated potassium channels ($SK_{(Ca)}$) (Vogalis & Goyal, 1997). As the RMP of SMCs is above the equilibrium potential for potassium channels, their opening causes an outward current of positive charges that hyperpolarizes the RMP and moves the SMC away from the opening of calcium channels necessary for contraction.

Motor neurotransmission in the GI tract occurs via specialized synapses with interstitial cells that lie between enteric nerve terminals and SMCs. $P2Y_1$ receptors are expressed both in SMCs and in PDGFRα+ cells (fibroblast-like cells) (Iino *et al.*, 2009). It has been recently reported that PDGFRα+ cells contain the apparatus to transduce purinergic signals (Iino & Nojyo, 2009;Cobine *et al.*, 2011;Kurahashi *et al.*, 2011;Iino *et al.*, 2009;Fujita *et al.*, 2003;Vanderwinden *et al.*, 2002). Moreover, calcium imaging experiments have also showed that PDGFRα+ cells increase their calcium levels just after purinergic neurons and before SMCs (Baker *et al.*, 2015). Thus, PDGFRα+ cells may mediate purinergic responses (Figure 10), and the hyperpolarization is then transmitted to SMCs through gap junctions. This organization would explain why a response involving IP_3 and calcium increase does not produce contraction.

Nitrergic neurotransmission

In 1990, Bult et al. proposed nitric oxide (NO) as the other NANC inhibitory neurotransmitter mediating smooth muscle relaxation in the gastrointestinal tract (Bult *et al.*, 1990). NO is a molecule produced by a family of enzymes called NO synthases (NOS), which produce NO from L-arginine. There are three genes encoding for NOS, the neuronal (nNOS) is the one responsible for NO production in the neurons of the gastrointestinal tract (Keef *et al.*, 1993;Sanders & Ward, 1992;Brookes, 1993;Lefebvre, 1993;Lefebvre *et al.*, 1995;Rand & Li, 1995).

NO is lipophilic and therefore diffuses through the plasma membrane and signals intra and intercellularly. The intracellular pathway for NO is mediated by soluble guanylyl cyclase (GC) (Waldman & Murad, 1987). This enzyme synthesizes cyclic guanosine monophospate (cGMP) from guanosine triphosphate (GTP) (De Man *et al.*, 2007). cGMP activates a protein kinase G (PKG), generating a cascade of phosphorylations, including the phosphorylation of potassium channels. PKG can also activate the MLCP promoting relaxation (Wang *et al.*, 2005). Both L-NNA (a NOS inhibitor) and ODQ (a GC blocker) inhibit the slow component of the IJP indicating that NO is responsible for the slow hyperpolarization observed in SMCs when inhibitory neurons are stimulated. As in many regions of the GI tract, colonic myogenic activity is tonically suppressed by the release of NO, maintaining an inhibitory tone. This can be also observed *in vitro* since TTX, L-NNA or ODQ cause a similar increase in motility (Gil *et al.*, 2010).

Junctional specializations exist between enteric nerve terminals and ICCs (Beckett *et al.*, 2005;NELEMANS & NAUTA, 1951;Burns *et al.*, 1996). Cholinergic

and nitrergic innervation results from the activation of specific receptors and signaling pathways in ICC-IM that are then conducted to neighboring SMCs (Burns *et al.*, 1996;Iino *et al.*, 2008;Ward & Sanders, 2001) (Fig 10). Recent studies have shown that for a correct nitrergic response, GC must be present in both SMCs and ICCs (Lies *et al.*, 2015)

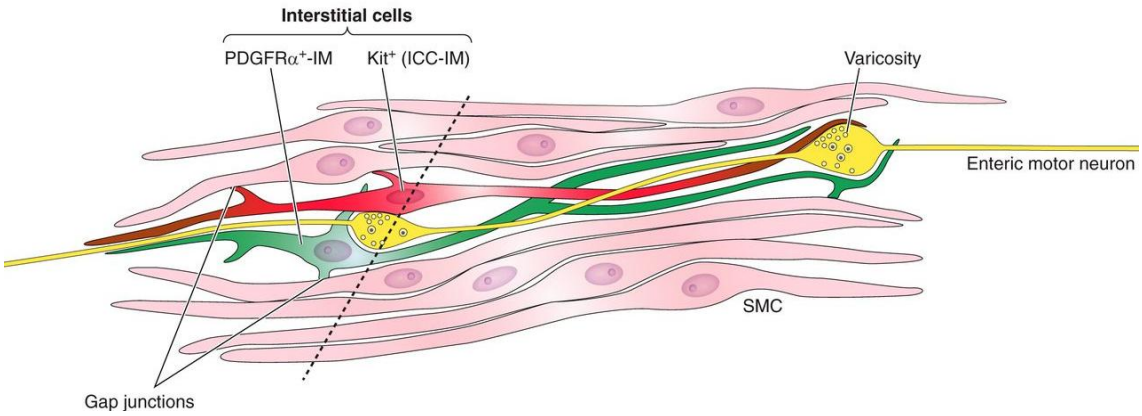

Figure 10. Smooth muscle cells, ICC, and PDGFRα+ cells are arranged around projections of excitatory and inhibitory enteric motor neurons (Sanders *et al.*, 2014).

Co-transmission

Burnstock postulated already in 1976 that some nerve fibers can synthesize, store and release more than one nerve transmitter (Burnstock, 1976). In 1994, coexistence of ATP and NO was reported in myenteric neurones of the rat colon using the quinacrine fluorescence technique to detect ATP (Belai & Burnstock, 1994). Since then, the existence of NANC inhibitory nerves that contain a combination of ATP and NO has been assumed by the scientific community (Furness *et al.*, 1989). Since ATP is a heavily charged molecule, a specific transporter is needed to facilitate its uptake (Schlafer *et al.*, 1994;Stadler & Fenwick, 1983). Recently, SLC17A9 has been proposed to be the transporter responsible for filling purinergic vesicles (Chaudhury *et al.*, 2012).

This has allowed the identification of vesicles containing ATP in nitrergic neurons, although ATP has been shown to be stored in all types of synaptic vesicles co-packaged with other neurotransmitters. However, there is abundant evidence that both ATP and NO are released stimulation-dependently from nerve endings upon depolarizing stimuli in the gastrointestinal tract (Figure 11).

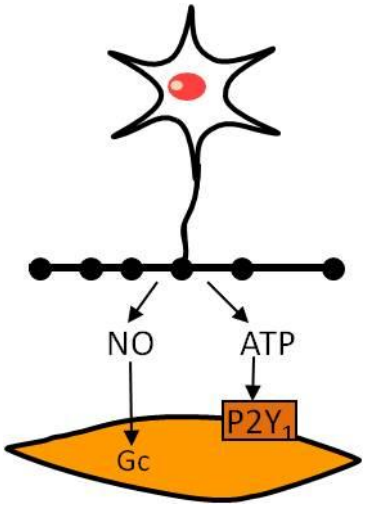

Figure 11. Enteric inhibitory neuron releasing both purines and NO. ATP evokes IJPf through activation of $P2Y_1$ receptors. NO causes IJPs through activation of Gc.

AIMS

The aims of the present thesis were the following:

- To characterize the effect of the novel $P2Y_1$ antagonist BPTU on the purinergic responses in the gastrointestinal tract of rodents. BPTU has been tested as an antithrombotic agent and has proven to have safety advantages over other antithrombotic drugs. However, the role of $P2Y_1$ receptors in gastrointestinal relaxation makes it compulsory to test if BPTU is also able to reach the neuromuscular junction in the gastrointestinal tract.
- To characterize the dynamics of purinergic-nitrergic neurotransmission in the human, rat and murine colon. Although both neurotransmitters are inhibitory and cause relaxation, the functional role of each co-transmitter has not been yet well established. We will study the different conditions that lead to the release of one or another transmitter and the behaviour of each response with the different parameters of neuronal firing.
- To study the differences of inhibitory co-transmission along the colon in the mouse. The proximal and distal colon display different main physiological roles. The aim is to determine if differences in the co-transmission process, together with the anatomic differences, can explain this functional divergence.
- To study the interaction of inhibitory neurotransmission and pacemaker activity in the rat colon. Enteric nerves and ICCs are extremely close to each other and even form specialized junctions. We will study how inhibitory neurotransmission affects each pacemaker in detail.
- To analyze the possible electrophysiological basis of the motility patterns displayed in vivo by the rat and human colon. A big gap still exists between motility in vitro experiments and in vivo recordings using manometry.

Therefore, we will try to correlate the patterns observed in vitro with the ones observed in vivo taking into account their characteristics and pharmacology.

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PUBLICATIONS

CHAPTER 1

Dynamics of inhibitory co-transmission, membrane potential and pacemaker activity determine neuromyogenic function in the rat colon

Pflugers Arch, **466**, 2305-2321.

Pflugers Arch - Eur J Physiol (2014) 466:2305–2321
DOI 10.1007/s00424-014-1500-8

ORGAN PHYSIOLOGY

Dynamics of inhibitory co-transmission, membrane potential and pacemaker activity determine neuromyogenic function in the rat colon

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Received: 30 January 2014 / Revised: 26 February 2014 / Accepted: 10 March 2014 / Published online: 25 March 2014

Abstract Interaction of different neuromyogenic mechanisms determines colonic motility. In rats, cyclic depolarizations and slow waves generate myogenic contractions of low frequency (LF) and high frequency (HF), respectively. Interstitial cells of Cajal (ICC) located near the submuscular plexus (SMP) generate slow waves. Inhibitory junction potential (IJP) consists on a purinergic fast (IJPf) followed by a nitrergic slow (IJPs) component leading to relaxation. In the present study, we characterized (1) the dynamics of purinergic-nitrergic inhibitory co-transmission and (2) its contribution on prolonged inhibition of myogenic activity. Different protocols of electrical field stimulation (EFS) under different pharmacological conditions were performed to characterize electrophysiological and mechanical responses. Smooth muscle cells (SMCs) in tissue devoid of ICC-SMP had a resting membrane potential (RMP) of −40.7±0.7 mV. Single pulse protocols increased purinergic and nitrergic IJP amplitude in a voltage-dependent manner ($IJPf_{MAX}$=−26.4±0.6 mV, $IJPs_{MAX}$=−6.7±0.3 mV). Trains at increasing frequencies enhanced nitrergic (k=0.8±0.2 s, $IJPs_{\infty}$=−15±0.5 mV) whereas they attenuated purinergic responses (k=3.4±0.6 s,$IJPf_{\infty}$=−8.9±0.6 mV). In tissues with intact ICC-SMP, the RMP was −50.0±0.9 mV and nifedipine insensitive slow waves (10.1±2.0 mV, 10.3±0.5 cpm) were recorded. In these cells, (1) nitrergic and purinergic responses were reduced and (2) slow waves maintained their intrinsic frequency and increased their amplitude under nerve-mediated hyperpolarization. Based on the co-transmission process and consistent with the expected results on RMP, prolonged EFS caused a progressive reduction of LF contractions whereas HF contractions were partially insensitive. In conclusion, inhibitory neurons modulate colonic spontaneous motility and the principles determining post-junctional responses are (1) the frequency of firing that determines the neurotransmitter/receptor involved, (2) the transwall gradient and (3) the origin and nature of each myogenic activity.

Keywords ICCs · Purinergic-nitrergic co-transmission · Inhibitory junction potential · Transwall gradient

Abbreviations

ADPβS	Adenosine 5′-O-2-thiodiphosphate
β-NAD	β-Nicotinamide adenine dinucleotide
CO	Carbon monoxide
EFS	Electrical field stimulation
ENS	Enteric nervous system
HF	High frequency
ICC	Interstitial cells of Cajal
IJP	Inhibitory junction potential
KO	Knockout
LF	Low frequency
L-NNA	N^{ω}-Nitro-L-arginine
MRS2500	(1R,2S,4S,5S)-4-[2-Iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicycle [3.1.0]hexane-1-methanol dihydrogen phosphate ester diammonium salt
NANC	Non-adrenergic non-cholinergic

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NO	Nitric oxide
NOS	Nitric oxide synthase
ODQ	Oxadiazolo[4,3-α]quinoxalin-1-one
PDGRFα+ cells	Fibroblast-like cells
RMP	Resting membrane potential
SEM	Standard error of the mean
SMC	Smooth muscle cell

Introduction

Intestinal smooth muscle cells (SMCs) are under the control of different mechanisms of regulation. The intertwined action of the networks of interstitial cells of Cajal (ICCs) and the enteric nervous system (ENS) determines, in great measure, the motor patterns observed in the colon. As enteric neurotransmission [20, 24] and pacemaker function [31, 45, 46] have been previously well characterized in the rat colon, this model has been used in the present work to study the interaction between inhibitory neurons and myogenic activity.

A co-transmission process is a general mechanism of neuromuscular interaction present in peripheral neurons including those located in the ENS [5]. However, little is known about how post-junctional responses vary depending on how neurons are stimulated. A well-known example of a co-transmission process is "fast and slow co-transmitters" that classically includes a neuropeptide. Single presynaptic action potentials release small molecule neurotransmitters, but trains of impulses are needed to release neuropeptides [6]. Enteric inhibitory motor neurons mainly encode for ATP or a related purine [7, 44] and nitric oxide (NO) [4]. Release of such inhibitory neurotransmitters produces a hyperpolarization or inhibitory junction potential (IJP) in SMCs when these neurons are stimulated. It is well known that single pulses or short trains of electrical field stimulation (EFS) induce a biphasic IJP—a fast (IJPf) followed by a slow (IJPs) component [11, 16, 29]. Recently, it has been demonstrated that the IJPf is blocked by $P2Y_1$ antagonists (MRS2179, MRS2279 and MRS2500) and, therefore, it is purinergic mediated [18, 24] while the IJPs is sensitive to NO inhibitors (L-NNA and ODQ) [21, 40, 46]. Moreover, $P2Y_1$ knockout (KO) animals lack purinergic IJPf and have intact nitrergic IJPs [17, 22, 33], confirming previous pharmacological approaches. Both putative co-transmitters hyperpolarize SMCs and are able to modify myogenic spontaneous activity.

The resting membrane potential (RMP) of SMC is graded across the circular muscle layer. This phenomenon has been defined as a "transwall gradient" and has been observed in the gastrointestinal tract of dogs [3, 26, 55], cats [57], mice [14, 53, 54] and humans [26]. In the mouse colon, SMCs next to the submucosa are comparatively more hyperpolarized to those located close to the myenteric plexus possibly due to the generation of carbon monoxide (CO) from submucosal ganglion neurons [54]. Thus, the colon is a good model to check how the excitability level of SMCs can affect post-junctional responses.

ICCs act as pacemaker cells in the gut [32, 58, 61] and in other tissues where myogenic rhythmic activity is required [28, 47, 52]. In the rat colon, classical slow waves originated in the network of ICC located in the submuscular (ICC-SMP) overlap with rhythmic and cyclic myogenic depolarizations [1, 45]. Accordingly, in the organ bath, circularly oriented colonic strips display low-frequency (LF) (0.5–1.5 cpm), high-amplitude contractions associated with cyclic depolarizations, superimposed to low-amplitude, high-frequency (HF) (13–15 cpm) contractions that correspond to slow waves. This electromechanical coupling is possibly the basis of the myogenic motor patterns observed with video recordings of colonic segments [31]. In vivo, electromyographic recordings [15] and colonic motility monitored with strain gauges transducers [39] reveal the presence of cyclic spike bursts (0.5–1 cpm) causing "giant migrating contractions" at an equivalent frequency to cyclic depolarizations and LF contractions recorded in vitro. Therefore, under different experimental conditions, it is clear that two types of electrical and mechanical phenomena are responsible for colonic motor function.

The aim of this paper is to study the interaction between inhibitory neurons and myogenic activity in the rat colon taking into account (1) the dynamics of the nitrergic and purinergic co-transmission process, (2) the excitability of SMC that might be conditioned by the transwall gradient and (3) the origin and nature of each myogenic activity. For this purpose, we used different parameters (voltage and frequency) of EFS and specific receptor or pathway blockers to isolate the contribution of each neurotransmitter to colonic SMC hyperpolarization and effect on colonic spontaneous contractility.

Materials and methods

Animals and tissue samples

Male Sprague-Dawley rats aged 8–10 weeks old, weighing 300 to 350 g, were kept at a constant room temperature of 22± 2°C and 55±10 % humidity, with a 12-h light:12-h dark cycle and ad libitum access to water and food. Animals were stunned and killed by decapitation and exsanguination. The colon was quickly removed and placed in carbogenated (95 % O_2 and 5 % CO_2) physiological Krebs solution. The mesenteric fat was removed; the mid-colon was opened along the mesenteric border and pinned to a Sylgard base with the mucosa facing upwards. Mucosal and submucosal layers were carefully removed for microelectrode experiments. For organ bath experiments and for the microelectrode protocols where

slow waves needed to be recorded, only the mucosal layer was removed. Transmural mid-colon muscle strips of 3-mm wide by 10-mm long were cut in the direction of the circular muscular fibres. All procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona.

Intracellular microelectrode recording

Muscle strips were pinned to a Sylgard-coated chamber, circular muscle or submucosa facing upwards and continuously perfused with carbogenated Krebs solution at 37±1ºC. Strips were allowed to equilibrate for approximately 1 h before experiments were undertaken. Circular SMCs were impaled with glass microelectrodes filled with 3 M KCl (30–60 MΩ of resistance). Membrane potential was measured using a standard electrometer Duo773 (WPI Inc., Sarasota, FL, USA). Tracings were displayed on an oscilloscope 4026 (Racal-Dana Ltd., Windsor, England) and simultaneously digitalized (100 Hz) with PowerLab 4/30 system and Chart 5 software for Windows (both from ADInstruments, Castle Hill, NSW, Australia). Intramuscular nerves were stimulated (pulse duration 0.3 ms; variable voltage, from 8 to 40 V and supramaximal voltage at variable frequency, from 0.1 to 10 Hz) using two silver chloride plates placed perpendicular to the longitudinal axis of the preparation and 1.5 cm apart. To obtain stable impalements, the tissue was perfused with nifedipine (1 μM) to abolish mechanical activity. RMP was measured at the bottom of slow wave activity.

Mechanical studies

Muscle strips were mounted in a 10-mL organ bath containing carbogenated Krebs solution at 37±1ºC. Circularly orientated preparations were tied to a support at one end and to an isometric force transducer (Harvard VF-1, Harvard Apparatus Inc., Holliston, MA, USA) at the other using a 2/0 silk thread. The transducer was connected to a computer through an amplifier, and data were digitalized (25 Hz) using Data 2001 software (Panlab, Barcelona, Spain) coupled to an A/D converter. A tension of 1 g was applied, and the tissue was allowed to equilibrate ≥1 h before performing any experiments. In order to study the interaction between inhibitory neurotransmitter release and ICC pacemaker activity, EFS was applied through almost all the experimental protocol (pulse duration, 0.3 ms; frequency, 5 Hz, increasing voltages from 1 to 20 V) through two platinum electrodes placed on the support holding the tissue. The effect of EFS and drug addition on the amplitude and frequency of both pacemakers was measured.

Solutions and drugs

The composition of the Krebs solution was (in mM): glucose, 10.10; NaCl, 115.48; $NaHCO_3$, 21.90; KCl, 4.61; NaH_2PO_4, 1.14; $CaCl_2$, 2.50 and $MgSO_4$, 1.16 (pH 7.3–7.4). The Krebs solution (37±1 °C) was bubbled with carbogen (95 % O_2 and 5 % CO_2). Propranolol, phentolamine and atropine (1 μM) were added to the Krebs solution to block β- and α-adrenoceptors and muscarinic receptors, respectively, in the microelectrode experiments. The following drugs were used: atropine sulphate, nifedipine, N^{ω}-nitro-L-arginine (L-NNA), phentolamine, sodium nitroprusside (SNP) 1H-[1, 2, 4] and oxadiazolo[4,3-α]quinoxalin-1-one (ODQ) (Sigma Chemicals, St. Louis, MO, USA), propranolol and (1R,2S,4S,5S)-4-[2-Iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicycle [3.1.0]hexane-1-methanol dihydrogen phosphate ester diammonium salt (MRS2500) (Tocris, Bristol, UK). Stock solutions were made by dissolving drugs in distilled water, except for nifedipine and ODQ, which were dissolved in 96 % ethanol and L-NNA, which was dissolved in Krebs solution by sonication.

Data analysis and statistics

Data are expressed as mean±SEM. Non-linear regression was used to fit experimental data (X=voltage or frequency of EFS vs. Y=amplitude of electrophysiological responses) to mathematical equations in microelectrode experiments. Linear regression was used on the increase of amplitude and frequency of slow waves with hyperpolarization. Student's unpaired t test was used to compare the differences in the IJPf decay between control and L-NNA-incubated tissue and between continuous and discontinuous stimulation. To normalize mechanical data, the effect of drugs and EFS was calculated as percentage of the original amplitude and frequency of both pacemakers, being 0 % when a total inhibition of pacemaker activity was recorded after drug administration or during EFS and 100 % when no changes in amplitude or frequency were observed. Two-way ANOVA was used to evaluate the effect of drugs or EFS on inhibition of amplitude and frequency of both pacemakers. Statistical analysis was performed with GraphPad Prism 5 for Windows (GraphPad Software, San Diego, CA, USA). Data were considered significant when $p<0.05$. "N" values represent samples from different rats.

Results

Purinergic and nitrergic IJP amplitude is reduced in the presence of slow waves

In tissue devoid of mucosa and submucosa (no SMP) incubated with non-adrenergic non-cholinergic (NANC) Krebs, single pulses (0.3 ms) elicited a biphasic IJP similar to the one represented in Fig. 1a. The duration of the IJPf (a) was 0.8 ±0.1 s while the IJPs (b) lasted about 8.0±1.1 s (N=10). Isolated purinergic IJPf (MRS2500-sensitive) were elicited

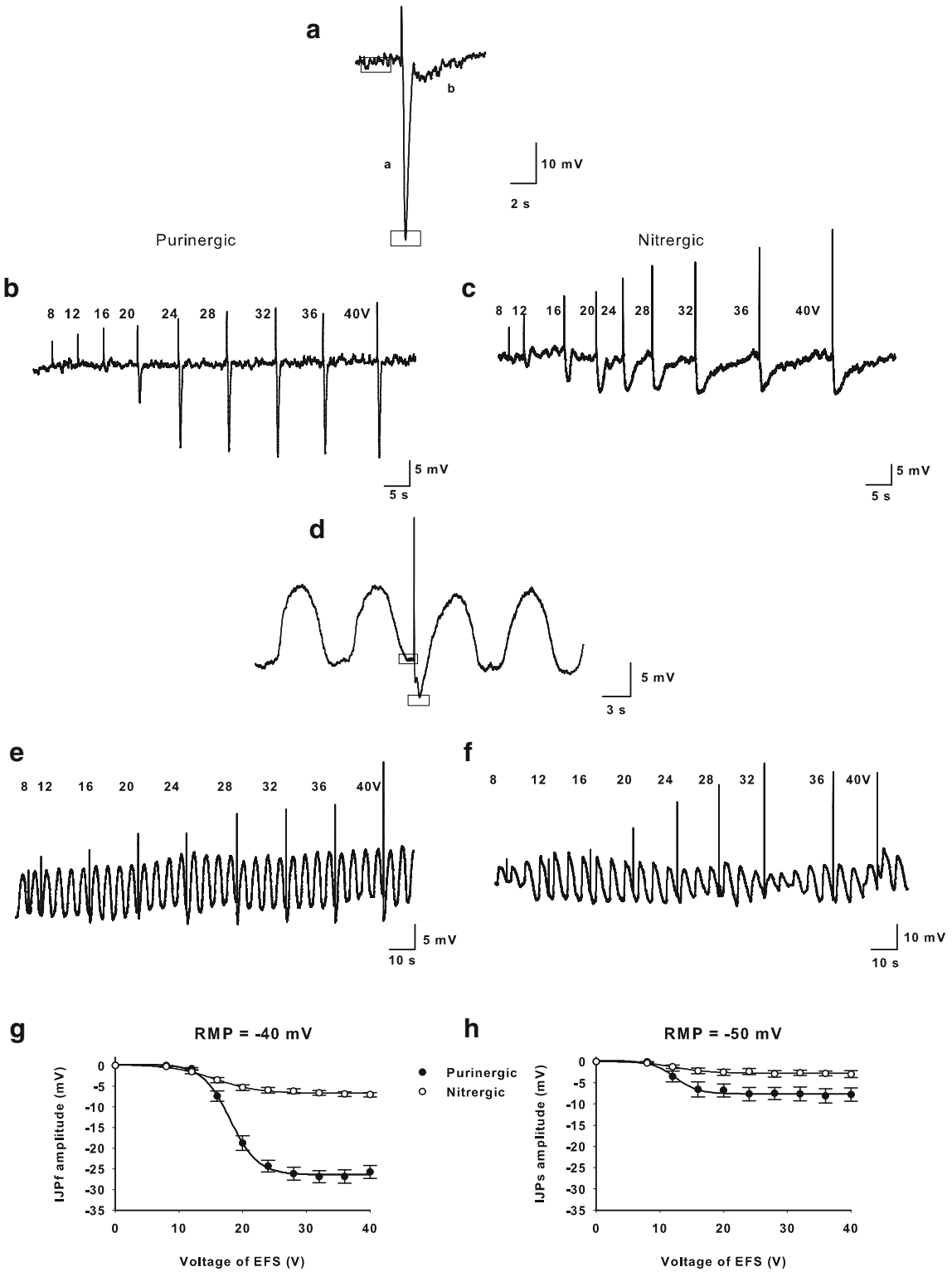


Fig. 1 **a** Single pulse stimulation in NANC Krebs elicits a biphasic IJP composed of a IJPf (*a*) followed by a IJPs (*b*). The amplitude of the IJP is measured from box to box. Microelectrode recording of a voltage-increasing stimulation protocol in L-NNA (1 mM) (**b**) and in MRS2500 (1 μM) incubated tissue with no SMP (**c**). **d** Single pulse of stimulation in tissue with intact SMP. The amplitude of the IJP is measured from box (*bottom of the slow wave*) to box. Microelectrode recording of a voltage-increasing stimulation protocol in L-NNA (1 mM) (**e**) and in MRS2500 (1 μM) incubated tissue with intact SMP (**f**). Experimental data fitted to a sigmoid voltage-response curve for both purinergic (Eq (1); *black dots*, DF=126, R^2=0.9; N=13) and nitrergic responses (Eq. (2); *white dots*, DF=176, R^2=0.6; N=18) in SMP devoid tissue (**g**) (RMP −40 mV) and strips with intact SMP (**h**) (RMP −50 mV) (Eq. (1); purinergic, DF=116, R^2=0.3; N=12; Eq. (2); nitrergic, DF=116, R^2=0.2; N=12). Notice the presence of the stimulus artefact just before the nerve mediated hyperpolarization

in colonic tissue incubated with L-NNA (1 mM) (Fig. 1b) and isolated nitrergic IJPs (L-NNA-sensitive) were obtained in the presence of MRS2500 (1 μM) (Fig. 1c). Single pulses at increasing voltages caused a progressive increase of both IJP amplitudes. Data (IJP amplitude vs. voltage of EFS) were fitted to a sigmoid voltage-response curve: (Eqs. 1 and 2) (Fig. 1g). Experimental values of the voltage equations are represented on Table 1.

Purinergic response to voltage

$$\mathrm{IJPf}_V(\mathrm{mV}) = \mathrm{IJPf}_{\mathrm{MAX}} - \frac{\mathrm{IJPf}_{\mathrm{MAX}}}{1 + 10^{(Vf50-V)\times \mathrm{Hill\ slope}(f)}} \quad (1)$$

Nitrergic response to voltage

$$\mathrm{IJPs}_V(\mathrm{mV}) = \mathrm{IJPs}_{\mathrm{MAX}} - \frac{\mathrm{IJPs}_{\mathrm{MAX}}}{1 + 10^{(Vs50-V)\times \mathrm{Hillslope}(s)}} \quad (2)$$

In tissue with intact SMP, impalements of SMCs next to the SMP showed the presence of nifedipine insensitive slow wave activity (amplitude 10.1±2.0 mV; frequency 10.3±0.5 cpm; N=7). Single pulses elicited an IJP similar to the one represented in Fig. 1d. The RMP measured at the bottom of the slow wave was −50.0±0.9 mV (N=20), while in SMP devoid tissue, the RMP was −40.7±0.7 mV (N=20) (Student's t test, significantly different, $p<0.0001$). This is consistent with the presence of a transwall gradient in the circular muscle layer.

Trains of increasing voltage in the presence of slow waves showed that both the purinergic and nitrergic IJP were approximately 60–70 % smaller in amplitude than those recorded in the absence of slow waves (Fig. 1e, f). This reduction in the amplitude of the response is probably due to the fact that the RMP is closer to the equilibrium potential when the cell is comparatively more hyperpolarized (Fig. 1h).

Increasing frequencies of stimulation enhances nitrergic and attenuates purinergic responses

Consecutive biphasic IJP were elicited at the supramaximal voltage increasing the frequency of stimulation from 0.1 to 10 Hz in NANC Krebs-incubated SMP devoid tissue (Fig. 2a). The fast component, which we considered to be the phasic hyperpolarizations, decreased its amplitude in a frequency-dependent manner, fitting an exponential decay. An exponentially increasing long-lasting hyperpolarization was recorded at high frequencies of stimulation. However, in these experimental conditions, it was not possible to distinguish the relative contribution of ATP and NO to each response (Fig. 2e). Therefore, we repeated the same protocol in (1) L-NNA (1 mM) incubated tissue (purinergic responses, Fig. 2b), (2) MRS2500 (1 μM) incubated tissue (nitrergic responses, Fig. 2c) and 3-MRS2500 (1 μM)+L-NNA (1 mM) incubated tissue (putative non-nitrergic non-purinergic responses, Fig. 2d). In L-NNA-incubated tissue, the slow component of the IJP disappeared and the IJPf decreased in a frequency-dependent manner as observed in control conditions (Student's unpaired t test, n.s.). A purinergic hyperpolarization of −8.9±0.6 mV remained at high frequencies of stimulation (IJPf_∞). The purinergic response was fitted to an exponential curve with a frequency-dependent decay (Eq. 3) (Fig. 2f). The time constant ($k(f)$) did not significantly differ with different IJPf initial amplitudes (not shown); therefore, the decay was independent of the amplitude of the IJPf. In MRS2500 (1 μM) incubated tissue, no IJPf was recorded. IJPs elicited an additive effect when increasing frequency of stimulation reaching −15.0±0.5 mV. The nitrergic hyperpolarization was fitted to the same exponential curve with a frequency-dependent increase in the response (Eq. 4) (Fig. 2g). The time constant ($k(s)$) did not significantly differ with different IJPs initial amplitudes (not shown); therefore, the increase was independent of the amplitude of the IJPs. In

Table 1 Purinergic and nitrergic responses depending on voltage of EFS in tissue devoid of the submuscular plexus (no SMP) and tissue with intact submuscular plexus (intact SMP). Experimental values of the parameters in the Eqs (1) and (2) that define the purinergic and nitrergic responses in SMCs at a RMP of −40 and −50 mV

	−40 mV (no SMP)	−50 mV (intact SMP)	Description
Purinergic response to voltage of EFSs			
	(N=13)	(N=12)	
$\mathrm{IJPf}_{\mathrm{MAX}}$	−26.4±0.6 mV	−7.6±0.6 mV	Maximum IJPf amplitude obtained with a single pulse
$Vf50$	18.0±0.4 V	12.3±1.4 V	Voltage at which the IJPf amplitude is half of the $\mathrm{IJPf}_{\mathrm{MAX}}$
Hill slope(f)	−0.20±0.03	−0.23±0.16	Hill slope of the fast component voltage-response curve
Nitrergic response to voltage of EFS			
	(N=18)	(N=12)	
$\mathrm{IJPs}_{\mathrm{MAX}}$	−6.7±0.3 mV	−2.7±0.3 mV	Maximum IJPs amplitude obtained with a single pulse
$Vs50$	15.7±1.0 V	12.5±2.5 V	Voltage at which the IJPs amplitude is half of the $\mathrm{IJPs}_{\mathrm{MAX}}$
Hill slope (s)	−0.13±0.03	−0.15±0.11	Hill slope of the slow component voltage-response curve

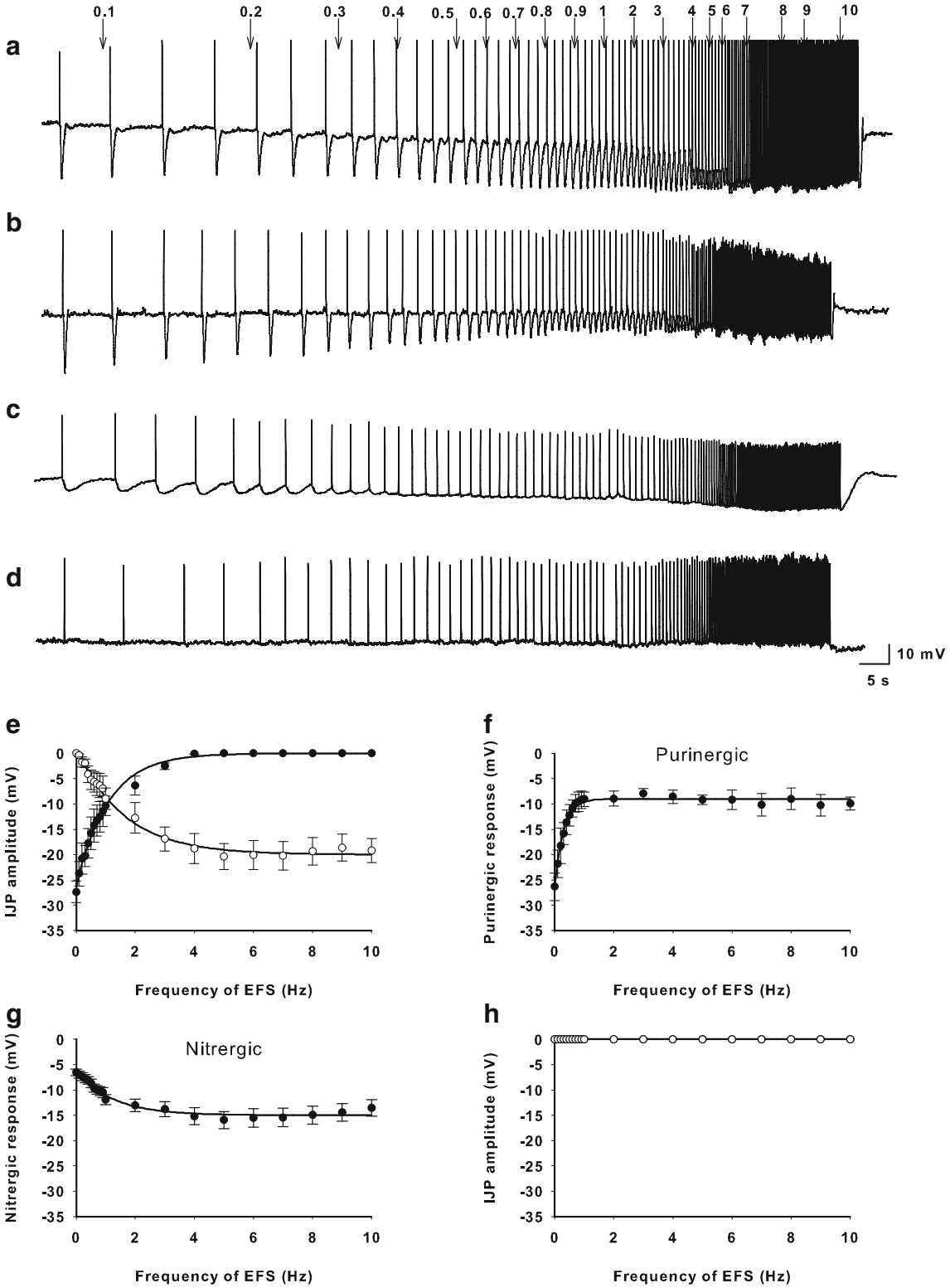


Fig 2 Microelectrode recording of tissue in control conditions (**a**), L-NNA (1 mM) incubated tissue (**b**), MRS2500 (1 μM) incubated tissue (**c**) and MRS2500 and L-NNA incubated tissue (**d**) stimulated at increasing frequencies of EFS (from 0.1 to 10 Hz). Notice the presence of the stimulus artefact in the recordings that helps to visualize the frequency of stimulation used. **e** Experimental data of the tissue in control conditions. IJPf amplitude fitted to an exponential frequency-dependent decay (*black dots*, DF=173, R^2=0.74; N=12) and hyperpolarization fitted to an exponential frequency-dependent increase curve (*white dots*, DF=164; R^2=0.74, N=12). **f** Purinergic response fitted to an exponential frequency-dependent decay curve (Eq. (3); DF=132, R^2=0.53; N=10) and **g** nitrergic response fitted to an exponential frequency-dependent increase curve (Eq. (4); DF=385; R^2=0.25; N=14). **h** No response was obtained in MRS2500 and L-NNA incubated tissue (N=14)

tissues incubated with both MRS2500 and L-NNA, no response to EFS was recorded (Fig. 2h), confirming the purinergic and nitrergic nature of the responses in this tissue. These results are summarized in Fig. 2, and the experimental values of the frequency equations are represented in Table 2.

Purinergic response to frequency

$$\mathrm{IJPf}_F(\mathrm{mV}) = (\mathrm{IJPf}_{\mathrm{MAX}} - \mathrm{IJPf}_\infty) \times e^{-k(f)F} + \mathrm{IJPf}_\infty \quad (3)$$

Nitrergic response to frequency

$$\mathrm{IJPs}_F(\mathrm{mV}) = (\mathrm{IJPs}_{\mathrm{MAX}} - \mathrm{IJPs}_\infty) \times e^{-k(s)F} + \mathrm{IJPs}_\infty \quad (4)$$

Comparison between protocols of continuous and discontinuous EFS

The previous protocols were performed increasing the frequency of stimulation in a continuous manner. However, as the purinergic response suffers a rundown, we tested if the measurements of the IJPf were also valid when isolated trains of stimulation (discontinuous stimulation) were applied. In this protocol, we elicited 20-s trains of a fixed frequency until reaching a stable response. Isolated trains were elicited at frequencies of 0.1, 0.3, 1, 3 and 5 Hz in L-NNA-incubated tissue (Fig. 3a). The response was a first IJPf followed by a purinergic response that rapidly diminished to a level maintained throughout the 20 s (Fig. 3b). The amplitude of the response for each frequency was measured in the steady state, and in these conditions, it did not differ from the one observed with continuous stimulation. The decay in the purinergic response with discontinuous stimulation, therefore, was not significantly different to the one obtained in continuous EFS (Student's unpaired *t* test, n.s.) (Fig. 3c).

Dynamics of purinergic-nitrergic co-transmission in the rat colon

According to the experimental data (Tables 1 and 2) the dynamics of purinergic-nitrergic co-transmission will depend on (1) the voltage of stimulation, (2) the RMP and (3) the frequency of stimulation. The fitting of experimental data to mathematical equations allowed us to generate two new equations able to predict the relative contribution of purinergic and nitrergic neurotransmission on inhibitory responses. Equations (1) (voltage-dependency) and (3) (frequency-dependency) were combined to predict the purinergic IJPf amplitude when any combination of voltage and frequency is elicited ($\mathrm{IJPf}_{F,V}$, Eq. (5)). Similarly, Eq. (2) (voltage-dependency) and (4) (frequency-dependency) were combined to predict the nitrergic IJPs amplitude ($\mathrm{IJPf}_{F,V}$, Eq. (6)). The co-transmission process is represented by the combination of Eqs. (5) and (6). Due to the difference in the time course of purinergic and nitrergic responses, the contribution of each neurotransmitter will vary depending on the frequency of EFS. At low frequencies, a biphasic IJP with the IJPf followed by the IJPs will be obtained (Fig. 1a). At high frequencies, however, the two components are no longer distinguishable due to an additive effect (Fig. 2a) and, therefore, the amplitude of the hyperpolarization will be the result of adding both responses. To be able to better understand the consequences of varying the voltage and frequency of stimulation, we graphically represented Eqs. (5) and (6) in Fig. 4. Notice that the results are now expressed in absolut values of RMP.

$$\mathrm{IJPf}_{F,V}(\mathrm{mV}) = \left((\mathrm{IJPf}_{\mathrm{MAX}} - \mathrm{IJPf}_\infty) \times e^{-k(f)F}\right) + \mathrm{IJP}_\infty) \times \left(1 - \frac{1}{1 + 10^{(Vf50 - V) \times \mathrm{Hillslope}(f)}}\right) + \mathrm{RMP} \quad (5)$$

$$\mathrm{IJPs}_{F,V}(mV) = \left((\mathrm{IJPs}_{\mathrm{MAX}} - IJPs_\infty) \times e^{-k(s)F}\right) + \mathrm{IJPs}_\infty) \times \left(1 - \frac{1}{1 + 10^{(V_{S50} - V) \times \mathrm{Hillslope}(s)}}\right) + \mathrm{RMP} \quad (6)$$

To generate these equations, we assume that (1) it can predict continuous and discontinuous protocols of stimulation (Fig. 3) and (2) due to the fact that the time constant for purinergic $k(f)$ and nitrergic $k(s)$ neurotransmission is independent of the amplitude of the IJP elicited with a single pulse, we assume that (a) the rundown/increase of

Table 2 Purinergic and nitrergic responses depending on the frequency of EFS in tissue devoid of the submuscular plexus. Experimental values of the parameters in the Eqs (3) and (4) that define the purinergic and nitrergic responses in SMCs at a RMP of −40 mV

Purinergic response to frequency of EFS (N=10)	Nitrergic response to frequency of EFS (N=14)	Description
$\mathrm{IJPf}_{\mathrm{MAX}}$=−26.4±0.6 mV	$\mathrm{IJPs}_{\mathrm{MAX}}$=−6.7±0.3 mV	Maximum IJP amplitude obtained with a single pulse
$k(f)$=3.4±0.6 s	$k(s)$=0.8±0.2 s	Time constant
IJPf_∞=−8.9±0.6 mV	IJPs_∞=−15.0±0.5 mV	Amplitude of the response obtained at high frequencies of stimulation

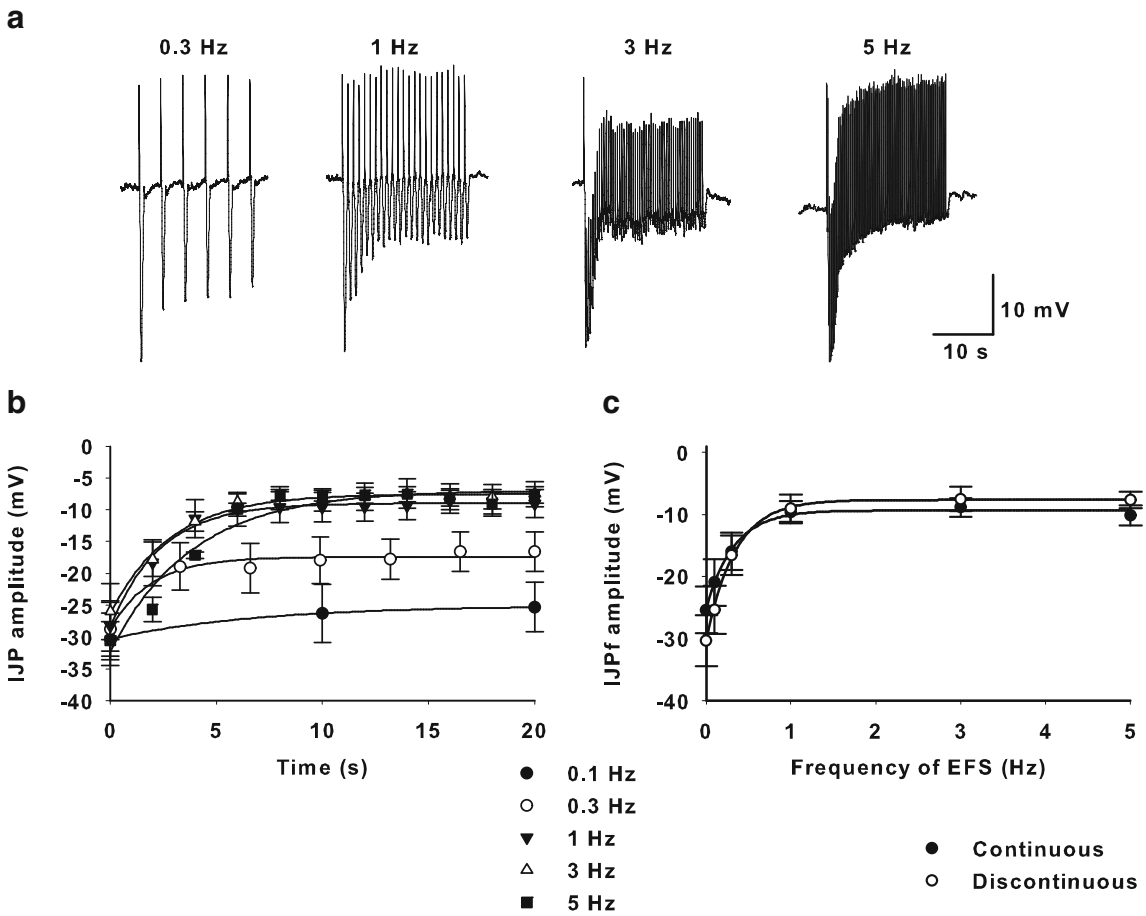


Fig 3 **a** Microelectrode recordings of 20-s trains of stimulation at frequencies of 0.3, 1, 3 and 5 Hz in L-NNA incubated tissue. **b** Representation of the amplitude of the purinergic IJP during the 20-s train of stimulation. **c** IJP amplitude measured during the steady state of the train fitted to an exponential frequency-dependent decay curve in discontinuous (Eq. (3); *white dots*, DF=21, R^2=0.7; N=4) and continuous (Eq. (3); *black dots*, DF=27, R^2=0.6; N=5) stimulation. Comparison of the time constants (k) revealed no significant differences between continuous and discontinuous protocols of EFS

the response will be proportional to the amplitude of the IJP elicited with a single pulse and (b) the IJP amplitude in SMCs at −50 mV will rundown/increase with the same time constant (Table 2).

Slow waves maintain their basal electrical activity under inhibitory neurotransmission

In order to study the interaction between inhibitory neurotransmission and slow wave activity, we hyperpolarized the tissue using EFS while recording slow waves (Fig. 5a). As SMCs are more hyperpolarized and the RMP is closer to the equilibrium potential, the response obtained was not higher than about −15 mV with both co-transmitters together. Hyperpolarization never stopped slow wave activity. Instead, as we diminished the membrane potential with inhibitory neurotransmitters release, slow waves increased their amplitude until reaching the same value of membrane potential at the top of the slow wave (Fig. 5b).

Inhibitory neurotransmission diminishes spontaneous mechanical activity

In the organ bath, tissues with intact SMP displayed low-amplitude HF contractions (0.25±0.04 g, 14.7±0.3 cpm) and high-amplitude LF contractions (4.3±0.5 g, 0.9±0.1 cpm). The HF contractions displayed in the organ bath were in the frequency range of the slow waves recorded with the microelectrode technique. Cyclic depolarizations corresponding to LF myogenic contractions can only be recorded with the microelectrode technique if no nifedipine is used [45].

We used constant stimulation at 5 Hz and increasing voltages of EFS to induce an inhibitory mechanical response. At the end of the protocol and during EFS, we reversed the mechanical inhibition with blockers of inhibitory neurotransmission (Figs. 6a and 7a). Spontaneous contractility progressively decreased when an increase in voltage of EFS was applied (Fig. 6a and b). The LF contractions markedly diminished with inhibitory neurotransmission (two-way ANOVA p<0.0001) (Fig. 6c). In contrast, HF contractions were less affected by inhibitory co-

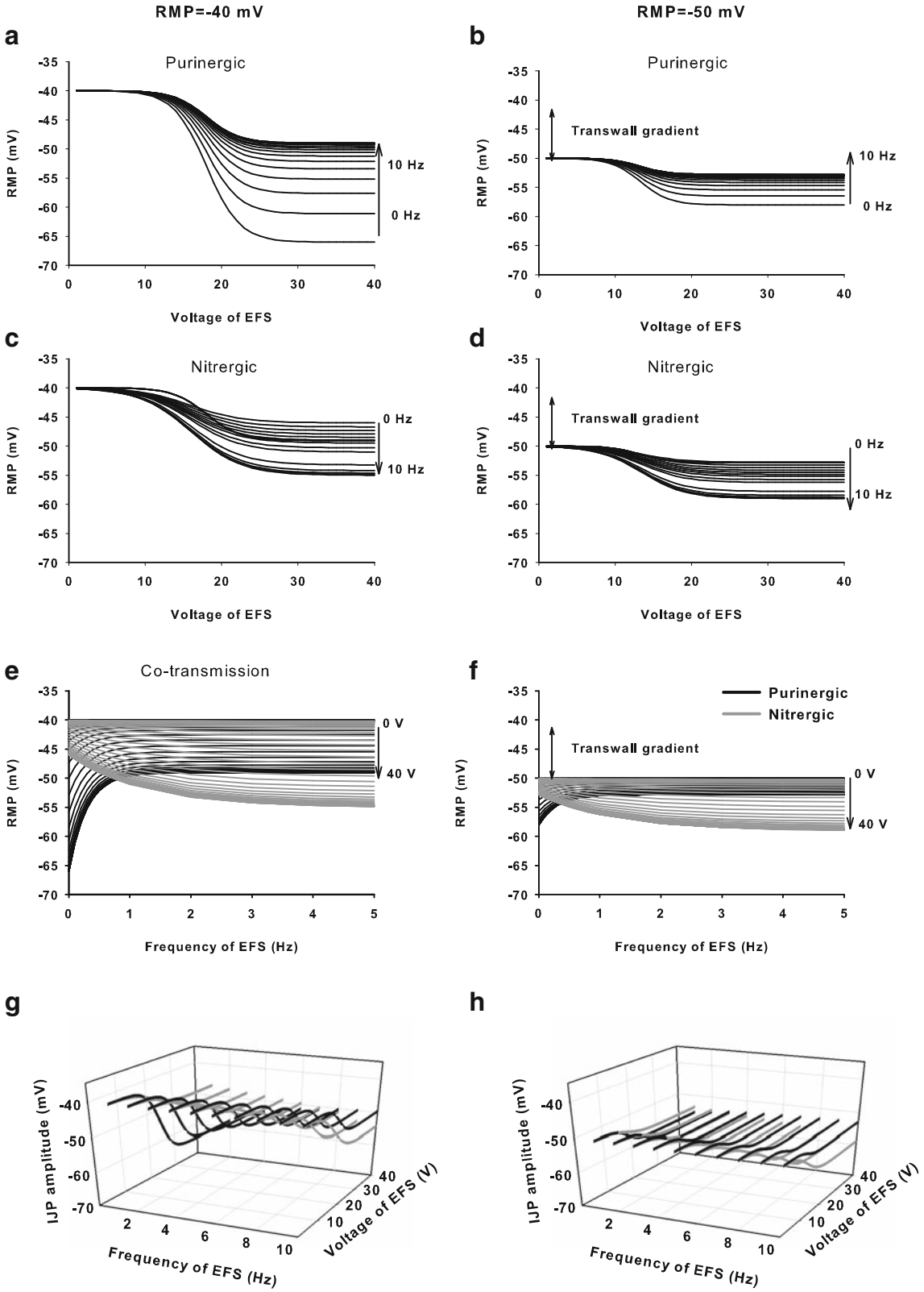


Fig 4 Inhibitory co-transmission in the rat colon. Effect of voltage at increasing frequencies of stimulation on post-junctional responses in SMCs with a RMP of −40 mV (**a** purinergic, **c** nitrergic) and with a RMP of −50 mV (**b** purinergic, **d** nitrergic). Effect of frequency at increasing voltages of stimulation in SMCs with a RMP of −40 mV (**e**) and with a RMP of −50 mV (**f**) on purinergic (*black*) and nitrergic (*grey*) responses. 3D representation of purinergic-nitrergic response (mV) vs. voltage and frequency of EFS in SMCs with a RMP of −40 mV (**g**) and with a RMP of −50 mV (**h**)

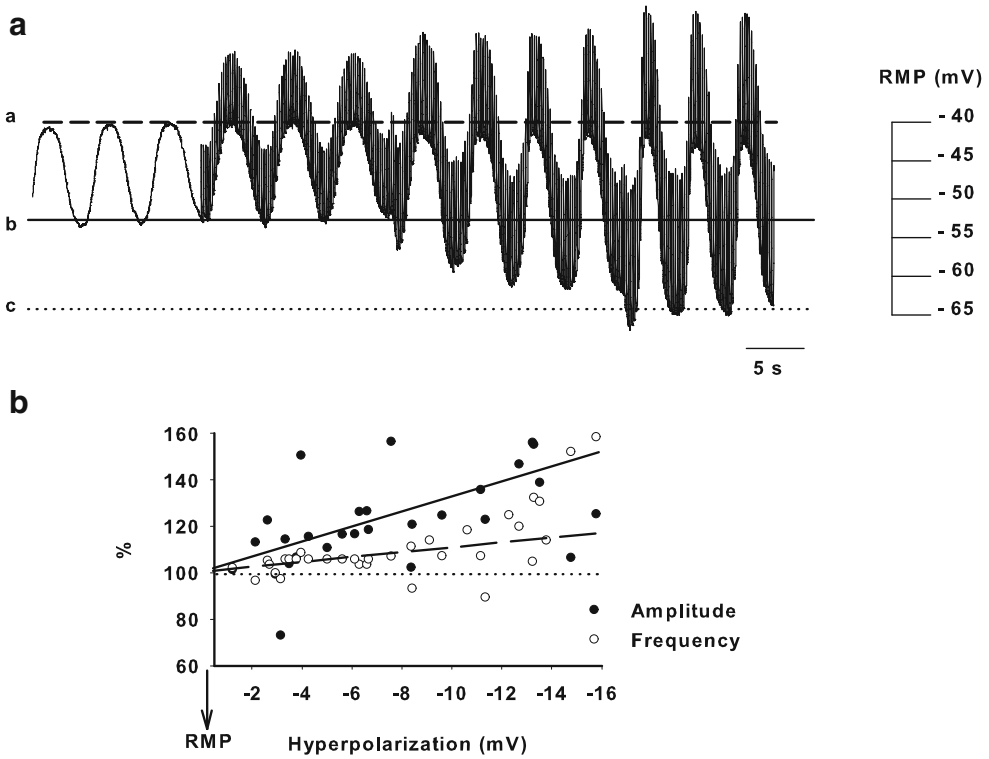


Fig 5 **a** Microelectrode recording of the effect of hyperpolarization on slow waves recorded near the SMP. Notice the presence of the stimulus artefact on top of the slow waves. "(*b*)" corresponds to the RMP measured at the bottom of slow wave activity and "(*a*)" to the top of the slow waves. Notice that when the tissue is hyperpolarized (c), slow waves increase their amplitude until reaching "(*a*)". **b** Graph with the effect of hyperpolarization on the amplitude (*solid line*) and frequency (*dashed line*) of slow waves. Linear regression showed a R^2 of 0.43 for amplitude and 0.49 for frequency with a slope significantly different from 0 ($p<0.0001$, $N=10$)

transmission (Fig. 6d) (two-way ANOVA n.s.). At high voltages of stimulation, a stimulatory response was often observed and therefore, in order to focus on inhibitory neurotransmission, we did not further increase the voltage of stimulation.

We used MRS2500 1 μM to isolate the effect of nitrergic neurotransmission on myogenic contractions. Isolated nitrergic neurotransmission was able to markedly inhibit LF contractions (two-way ANOVA $p<0.0001$) (Fig. 6e) but not HF contractions (Fig. 6f). The same effect was observed with the exogenous addition of increasing concentrations of sodium nitropusside (not shown, $N=6$). To revert nitrergic neurotransmission, ODQ 10 μM was added at the end of the protocol (Fig. 7b).

In order to test the effect of purinergic neurotransmission on spontaneous contractions, tissue was incubated with ODQ 10 μM. LF contractions were less affected by purinergic neurotransmission (Fig. 6g) compared to the prior protocols while HF contractions again proved to be insensitive to inhibitory neurotransmission. Purinergic neurotransmission was blocked at the end of the protocol with MRS2500 1 μM (Fig. 7c).

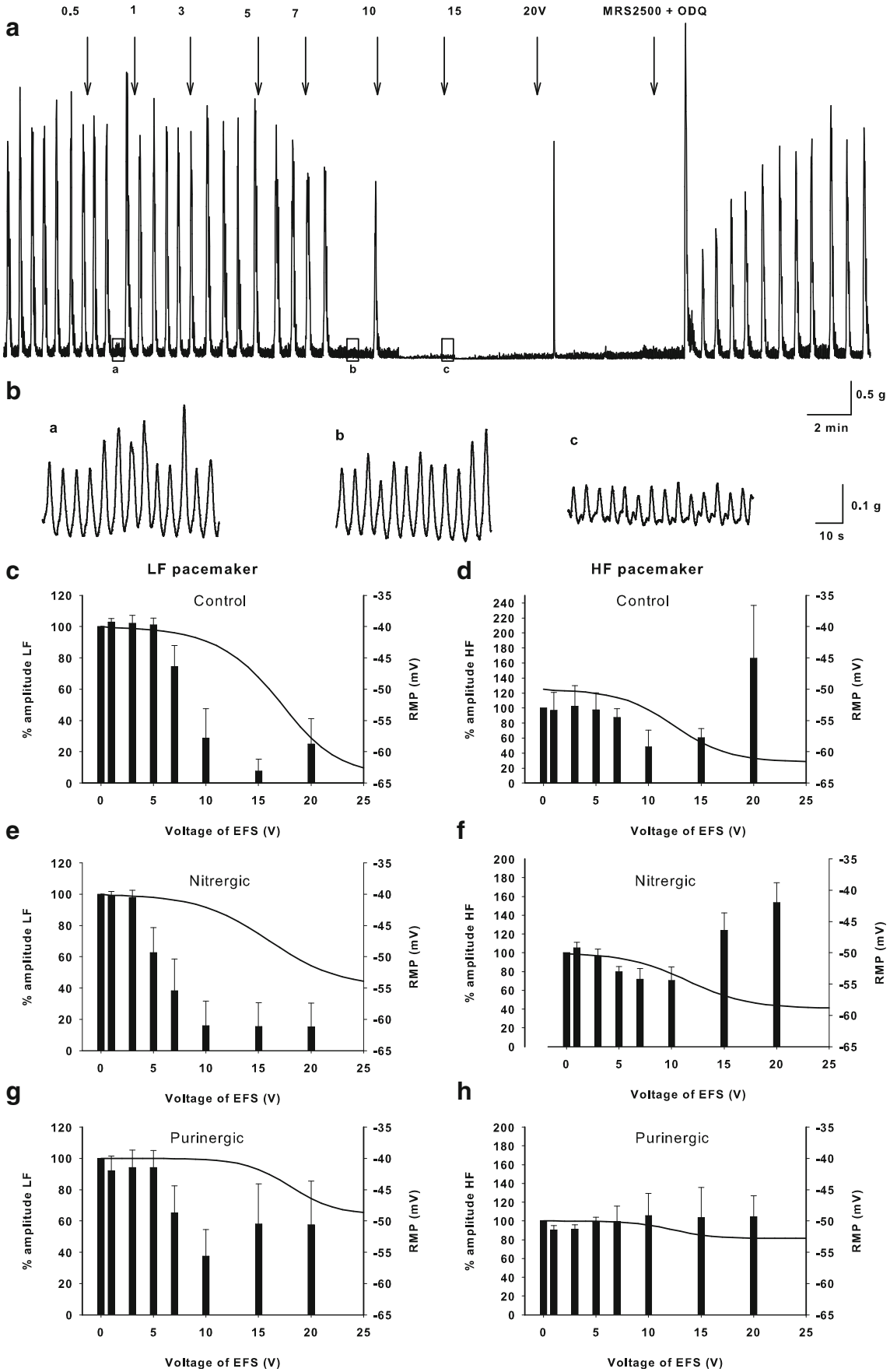
The effect of the 5-Hz continuous stimulation on RMP when an increase in voltage is applied at different levels of SMC excitability (RMP of −40 and −50 mV) was predicted using Eqs. (5) and (6). The hyperpolarization expected is represented with a line plotted vs. the right axis of the graphics in Fig. 6. In the graphics representing the inhibition of LF contractions, we used the expected response in SMCs with a RMP of −40 mV as it is likely to be the RMP in the area displaying this pacemaker. In contrast, the HF pacemaker responsible for HF contractions is originated in the SMP where the transwall gradient is created. Therefore, for HF contractions, we used the expected electrophysiological responses in SMC with a RMP of −50 mV.

The results obtained in spontaneous contractions were consistent with the expected changes in RMP. LF contraction amplitude diminished the most when both co-transmitters were inhibiting motility (Fig. 6c) and with nitrergic neurotransmission alone (Fig. 6e) as the hyperpolarization reached in these conditions is bigger. The expected purinergic hyperpolarization, however, is lower due to the rundown phenomenon present at 5 Hz and has less capacity to inhibit LF contractions in these circumstances (Fig. 6g). In the area where HF contractions are originated, the predicted hyperpolarization is lower and does not modify HF contractions amplitude.

Spontaneous contractility is restored during EFS with nitrergic and purinergic blockers

To verify that the inhibitory response on spontaneous contractility was attributable to each putative co-transmitter,

Fig 6 **a** Organ bath recording of both pacemakers and the effect of EFS ▶ at 5 Hz and increasing voltages on the LF pacemaker and reversion with MRS2500 and ODQ. In **b**, *a*, *b* and *c* correspond to the selected sections in **a**, recordings of the effect of inhibitory neurotransmission on the HF pacemaker. Effect of inhibitory neurotransmission elicited with uninterrupted 5-Hz EFS at increasing voltages on both pacemakers corresponds to the histogram and left *Y* axis while expected electrophysiological inhibitory responses are represented with a line and the right *Y* axis. The effect of both co-transmitters on pacemaker amplitude is represented in graphics **c** and **d** (control), effect of nitrergic neurotransmission in **e** and **f** and effect of purinergic neurotransmission in **g** and **h** ($N=6$)


a
0.5
1
3
5
7
10
15
20V
MRS2500 + ODQ
a
b
c
b
a
b
c
0.5 g
2 min
0.1 g
10 s
c
LF pacemaker
Control
% amplitude LF
RMP (mV)
Voltage of EFS (V)
d
HF pacemaker
Control
% amplitude HF
RMP (mV)
Voltage of EFS (V)
e
Nitrergic
% amplitude LF
RMP (mV)
Voltage of EFS (V)
f
Nitrergic
% amplitude HF
RMP (mV)
Voltage of EFS (V)
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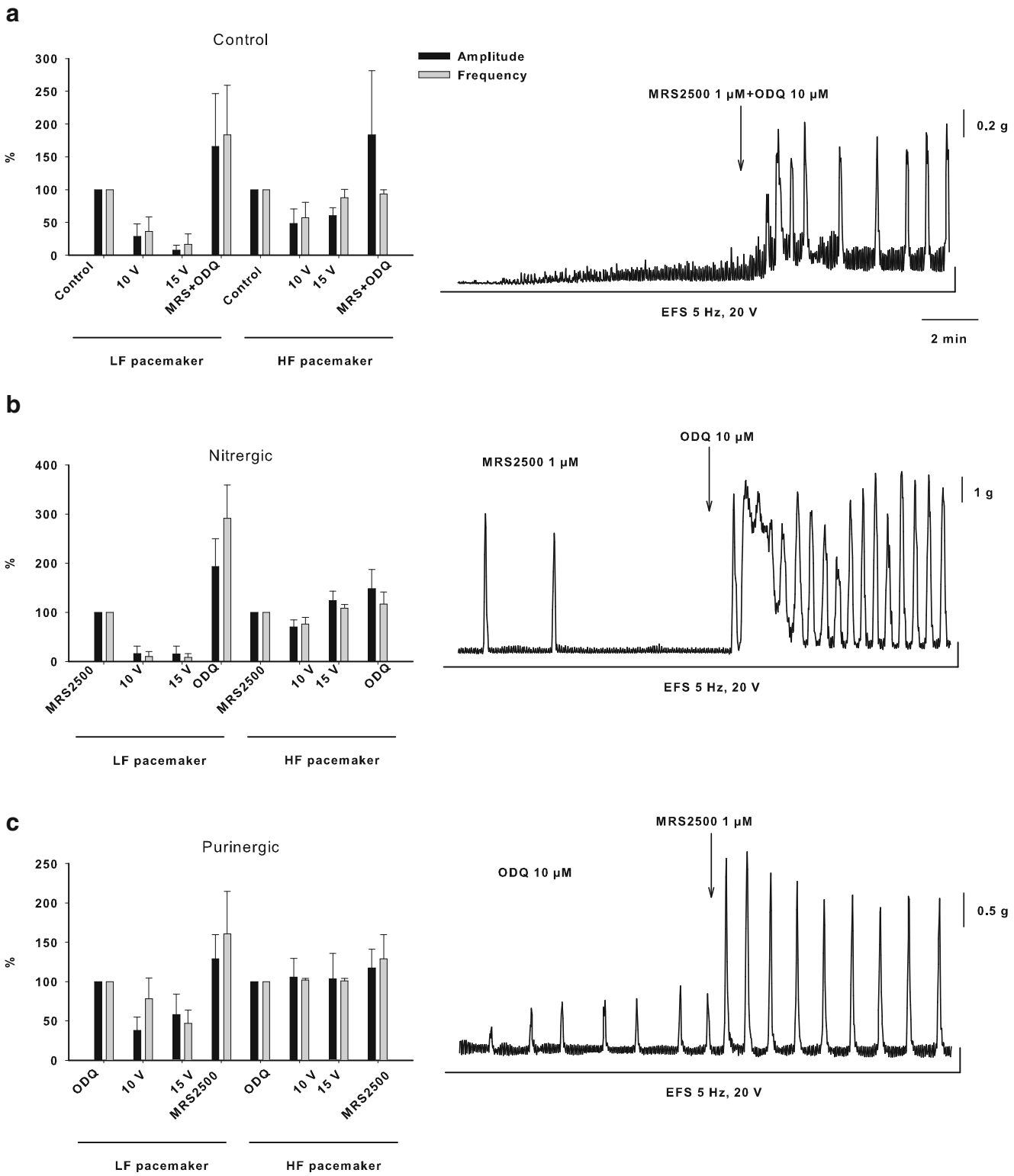

Fig. 7 Organ bath recordings (*right*) and graph (*left*) showing the effect of EFS on frequency and amplitude of both myogenic contractions. Inhibition and reversion values are expressed in percentages of basal amplitude and frequency of contractions (control). EFS at increasing voltage reduced both the amplitude and frequency of LF contractions and in each pharmacological protocol, myogenic activity is recovered during EFS. **a** Spontaneous motility in control conditions is reverted with MRS2500 and ODQ (N=6); **b** spontaneous motility in MRS2500 (1 μM) incubated tissue is reverted with ODQ (10 μM) and spontaneous motility in ODQ incubated tissue is reverted with MRS2500 (**c**). Notice that an enhanced myogenic activity compared to control is recorded in **a** and **b** due to the suppression of the nitrergic inhibitory neural tone

purinergic and nitrergic neurotransmission blockers were added at the end of the organ bath protocol without interrupting the 5-Hz stimulus at 20 V. Addition of MRS2500 (1 μM) and ODQ (10 μM) during EFS completely restored HF and LF contractions showing that both co-transmitters were responsible for the inhibitory effect on contractibility (Fig.7a, Fig. 6c, d). In the same way, ODQ (10 μM) added in tissue incubated with MRS2500 restored both motility patterns (Fig. 7b) confirming that the inhibitory effect on contractions was attributable to NO (Fig. 6e, f). Finally, MRS2500 added in ODQ incubated tissue during EFS also restored spontaneous contractions (Fig. 7c) confirming that the inhibition of contractions observed (Fig. 6g, h) was due to $P2Y_1$ activation.

Discussion

In the present work, we investigated the basic principles of the inhibitory co-transmission process in the rat colon and how neurotransmitters can cause a sustained inhibition of spontaneous myogenic contractions. We found that the crucial elements to properly characterize this phenomenon in vitro are (1) the voltage and frequency of EFS leading to neurotransmitter release that will determine the neurotransmitter and receptor involved in each response, (2) the transwall gradient in the RMP across the circular smooth muscle layer and (3) the nature of each pacemaker function and their associated spontaneous contractility.

As previously reported for the purinergic pathway [46], in the present work, we demonstrate that single pulses at increasing voltages of EFS cause higher nitrergic and purinergic post-junctional responses. This increase in the amplitude of both IJPf and IJPs is probably due to more recruited inhibitory neurons and/or more neurotransmitter being released from individual neurons. We mimicked the endogenous increase of neurotransmitter release by increasing the voltage of EFS in the organ bath and observed a progressive reduction of spontaneous myogenic contractions causing almost a cessation of spontaneous motility.

In contrast, in this work, we show how an increase in the frequency of EFS causes opposite effects between co-transmitters. When we progressively increase the frequency of stimulation from 0.1 to 10 Hz, we observed a decrease in the amplitude of the fast component of the IJP and, simultaneously, a progressive hyperpolarization that reached its maximum at high frequencies. Under these experimental conditions, it was not possible to distinguish the relative contribution of each neurotransmitter to the response. Therefore, we isolated the electrophysiological purinergic response by blocking NO synthesis with L-NNA and the nitrergic electrophysiological response using the $P2Y_1$ antagonist MRS2500. Notice that, in the rat colon circular muscle, at ranges of EFS frequencies from 0.1 to 10 Hz, all the inhibitory electrophysiological responses were abolished with the combination of L-NNA and MRS2500. Accordingly, in this tissue, inhibitory neuromuscular transmission is mainly due to purines and NO. In other tissues such as in the internal anal sphincter, the release of the vasointestinal peptide VIP has been demonstrated at high frequencies of stimulation (5 to 20 Hz) during long-duration trains [36].

With each response isolated, we observed that when we increased the frequency of stimulation, a progressive increase in the amplitude of nitrergic smooth muscle hyperpolarization was recorded. This increase due to NO occurs simultaneously to a progressive reduction of the MRS2500-sensitive IJPf amplitude that reaches a stable response at high frequencies (−8.9±0.6 mV). This latter phenomenon is known as "rundown" and has been described using two consecutive pulses of EFS in several parts of the gastrointestinal tract including the human colon [16, 37, 42]. In the present work, we have characterized and quantified the purinergic rundown in the rat colon, showing that the decay in the purinergic response is exponential ($k(f)$=3.4±0.6 s) when frequency of stimulation increases.

The reason for purinergic neurotransmission rundown is still unknown, and both pre- and post-junctional mechanisms could be responsible for this phenomenon. It is possible that at high firing frequencies the release of ATP stored in vesicles expressing SLC17A9 [8] of inhibitory neurons is attenuated due to neurotransmitter depletion. However, different purines (beta-NAD and ADP ribose) released by enteric inhibitory neurons have been measured with HPLC at 16 Hz of stimulation [13, 34, 44], which suggests that the amount of neurotransmitter is apparently not a limiting phenomenon to explain the reduction of purinergic responses. Another option is that a post-junctional mechanism is underlying the rundown. As previously demonstrated, $P2Y_1$ receptors are responsible for purinergic neurotransmission in the gastrointestinal tract [16, 17, 22, 24, 33]. $P2Y_1$ receptors are G protein-coupled and cause the release of calcium from intracellular stores through the IP_3 pathway when activated [19, 30]. Calcium activates small conductance calcium-activated potassium channels ($SK_{(Ca)}$) [2, 60] leading to hyperpolarization. This intracellular pathway, although still under debate [41, 63], could also be responsible for the post-junctional rundown. Moreover, several studies have shown that $P2Y_1$ receptor desensitization occurs due to phosphorylation of the receptor by PKC (dyacilglycerol-activated) [27, 50] and also that phosphorylation can lead to internalization of the $P2Y_1$ receptor after prolonged exposition to agonists [48]. Any of these mechanisms could be responsible for the decrease of purinergic

neuromuscular transmission when inhibitory neurons are stimulated at high frequency.

The increase in the amplitude of the nitrergic response when inhibitory neurons fire at relatively high frequencies follows the rules of a classical "temporal summation". In a co-transmission context, the NO would have a similar behaviour to neuropeptides in "fast and slow co-transmitters" as neuropeptides need high frequencies of stimulation to be released. In our case, however, it is unknown if inhibitory neurons are releasing one or another transmitter depending on their firing frequency as in the fast and slow co-transmitters process or post-junctional mechanisms are responsible for these differences. The NO effect is due to its action on guanylate cyclase (GC) [12, 21, 40], which causes smooth muscle hyperpolarization and inhibition of spontaneous contractility. In the present paper, we demonstrate that if continuous stimulation at high frequency is used, a sustained hyperpolarization of SMCs and, consequently, a tonic inhibition of spontaneous motility can be achieved. In contrast, enteric inhibitory motor neurons firing at low frequency release ATP or a related purine that transiently hyperpolarizes the smooth muscle causing a phasic (but not sustained) relaxation. We propose that in each gastrointestinal physiological process a predominant nitrergic/purinergic response will be needed in order to obtain a prevailing tonic/phasic relaxation. In conclusion, based on the co-transmission process defined by Burnstock in 1976 [5], in the present study, we demonstrate that depending on the number of enteric inhibitory neurons recruited and their putative endogenous firing frequency (7.3 ±4.5 Hz as suggested by Michel et al. [43]) different post-junctional responses and physiological functions will be elicited.

Our electrophysiological approach can be used in the study of human samples and animal models where inhibitory neuromuscular transmission is possibly impaired. To our understanding, the absence/impairment of nitrergic neurotransmission should never be demonstrated with protocols of single pulse stimulation. The increase in frequency will enhance nitrergic responses, and consequently, a frequency-dependent long-lasting response will better test the capacity to elicit NO release from enteric neurons. On the contrary, experiments to evaluate purinergic neurotransmission which can also be selectively unpaired [49, 56] should be performed with single pulses or short trains of stimulation because attenuation of purinergic responses will rapidly occur with prolonged stimulation at high frequencies.

Another important finding is the difference in RMP observed between SMCs from tissue with intact SMP and tissue without the SMP. Similarly to what was previously described by Sha and colleagues [54], our recordings in smooth muscle with intact SMP were about 10 mV more hyperpolarized. This is consistent with the presence of a transwall gradient along the thickness of the colonic muscle wall. To eliminate the transwall gradient, therefore, we removed the mucosa and submucosa layer. In these conditions, all cells should have the same RMP, the one expected in SMCs next to the ICC-MP [54]. In contrast, when our aim was to conserve the transwall gradient, only the mucosa layer was removed. In this case, to make sure that we obtained the data of cells adjacent to the SMP, we only performed the protocols in SMCs that displayed slow waves of a considerable amplitude indicative of proximity to the ICC-SMP. Both purinergic (~ −8 mV) and nitrergic (~ −3 mV) IJP amplitude were reduced in these preparations as cells are closer to the equilibrium potential. According to this phenomenon, the capacity to hyperpolarize SMCs near the SMP is considerably limited. In contrast, in the stomach and small intestine, the RMP of circular SMCs in the myenteric region is more hyperpolarized compared to the RMP of SMCs adjacent to the submucosa [3, 14, 26, 53, 57]. We hypothesize that the same phenomenon between inhibitory neurotransmission and pacemaker function is taking place in these regions of the gastrointestinal tract but in an inverted way. Interestingly, ICC-MP generates slow wave activity in these areas of the gastrointestinal tract. Therefore, in all cases, the region generating slow waves (ICC-MP in the stomach and intestine and ICC-SMP in the colon) is the most hyperpolarized and with less capacity to be further hyperpolarized by inhibitory neurons.

Combination of slow waves and cyclic depolarizations and their associated respective HF and LF spontaneous contractions determine myogenic motor patterns in the rat colon [31]. LF contractions are becoming increasingly important in colonic motility as it has been proven that, at least in some species, they are sufficient to produce colonic propulsion in the absence of neural activity [9, 10, 31]. In fact, this type of contractions strongly correlates to cholinergic "giant migrating contractions" [39] but, in vitro, they can possibly be evoked with stretch in the absence of a cholinergic input [31]. Cyclic depolarizations and the associated LF contractions are highly sensitive to L-type calcium channel blockers [45]. L-type calcium channels open at voltages above −40 mV in colonic SMCs, and consequently, inhibitory inputs causing smooth muscle hyperpolarization will inhibit contractile activity (present work). In contrast, rat colonic slow waves recorded near the ICC-SMP have properties similar to intestinal slow waves [35] and are relatively insensitive to nifedipine. Moreover, the transwall gradient in the RMP makes the capacity to hyperpolarize the cells near the SMP difficult. Furthermore, in the present study, we also demonstrate that when colonic tissue is hyperpolarized by inhibitory neurons, the amplitude of slow waves increases. This phenomenon was reported by Tomita in the guinea pig stomach when he showed how conditioning hyperpolarization (5–15 mV) increased the amplitude of the slow waves but had little effect on their frequency [35, 59] as seen in the present study. This supports the idea that this second pacemaker is relatively insensitive to

hyperpolarization as it has been shown when gaseous neurotransmitters such as H_2S are infused [23].

In the present paper, we demonstrate how purinergic and nitrergic inhibitory neurotransmission interact with both myogenic spontaneous contractions taking into account the RMP. According to our results, continuous stimulation of inhibitory neurons (5 Hz) is able to elicit the release of both NO and purines. In the area where the RMP is −40 mV, at 5 Hz of uninterrupted EFS, NO is expected to cause in the SMC ~15 mV hyperpolarization while the purine causes a ~10-mV remaining hyperpolarization due to the rundown phenomenon. In the region where slow waves are generated, this capacity to hyperpolarize would be reduced to ~−3 mV for the purinergic response and ~−11 mV for nitrergic response due to the transwall gradient. We demonstrated the capacity of inhibiting motility of both neurotransmitters together and isolated by blocking the other one during continuous EFS. We used MRS2500, a $P2Y_1$ blocker, and ODQ, a GC blocker, as a pharmacological approach. Inhibitory neurotransmitter release was able to inhibit LF contractions, while HF contractions persisted at high voltages. The greatest inhibition was obtained with both neurotransmitters together and when the nitrergic response was isolated as in these conditions, the predicted hyperpolarization was bigger. The purinergic component, however, was not able to elicit a significant inhibition of spontaneous contractions due to the purinergic rundown present at 5 Hz of continuous stimulation. After blocking both neurotransmitters during EFS, the normal motility pattern was restored, confirming that both $P2Y_1$ receptors and the GC pathway are causing motility inhibition. Interestingly, in some of the experiments, after adding the blocker, we observed a kind of "off-response" which was, in fact, a pharmacological off-response as the inhibitory response was finished by the blockade of inhibitory neurotransmission and not by interrupting EFS. This result confirms that the effect that we are observing in muscle bath experiments has the electrophysiological basis of the co-transmission process. Therefore, pacemaker activity is differently modulated by enteric inhibitory nerves because (1) the transwall gradient decreases the capacity to hyperpolarize SMCs where slow wave activity is recorded and (2) the voltage dependence of each pacemaker activity differs.

The organization and identity of the cells involved in the signalling of neuromuscular transmission in the gastrointestinal tract is complex and still under debate. Recently, PDGFRα+cells expressing $P2Y_1$ receptors have been proposed as crucial cells to mediate purinergic neuromuscular transmission [2, 38]. ICCs have been also postulated before as intercalated cells mediating nitrergic neurotransmission [62], and probably, GCs located both in the ICC and SMC contribute to nitrergic neurotransmission [25]. It is possible that part of the signaling pathway presented in this study occurs in PDGFRα+, ICC or SMCs consistent with the SMC/ICC/PDGFRα+syncitium proposed by Sanders and Ward [51]. Functional data presented in the present paper should be translated to the different areas of the human gastrointestinal tract in order to establish functional criteria to evaluate the possible impairment of neuromuscular transmission in human diseases.

Acknowledgments We thank Claudia Arenas, Antonio Acosta and Emma Martínez for their technical assistance.

Conflict of interest This work has been funded by the following grant: BFU2009-11118 and 2011 CTP 00032. Noemí Mañé (AP2012-0897), Víctor Gil (AP2007-01583) and Míriam Martínez-Cutillas (AP2010-2224) are supported by the Ministerio de Ciencia e Innovación (Spain). Diana Gallego is supported by the Instituto de Salud Carlos III, Centro de Investigación Biomédica en red de enfermedades hepáticas y digestivas (CIBERehd). The authors state no conflict of interest.

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CHAPTER 2

Differential functional role of purinergic and nitrergic inhibitory cotransmitters in human colonic relaxation

Acta Physiol (Oxf), **212**, 293-305.

ACTA PHYSIOLOGICA

Acta Physiol 2014, **212**, 293–305

Differential functional role of purinergic and nitrergic inhibitory cotransmitters in human colonic relaxation

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Received 31 July 2014,
revision requested 23 September 2014,
revision received 13 October 2014,
accepted 14 October 2014
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Abstract

Aim: ATP and nitric oxide (NO) are released from enteric inhibitory motor neurones and are responsible for colonic smooth muscle relaxation. However, how frequency of neural stimulation affects this cotransmission process and the post-junctional responses has not been systematically characterized in the human colon.

Methods: The dynamics of inhibitory cotransmission were studied using different protocols of electrical field stimulation (EFS) to characterize the inhibitory junction potentials (IJP) and the corresponding relaxation in colonic strips obtained from 36 patients.

Results: Single pulses elicited a fast IJP ($IJPf_{MAX} = -27.6 \pm 1.6$ mV), sensitive to the $P2Y_1$ antagonist MRS2500 1 μM, that ran down with frequency increase leaving a residual hyperpolarization at high frequencies ($IJPf_{\infty} = -3.7 \pm 0.6$ mV). Accordingly, low frequencies of EFS caused purinergic transient relaxations that cannot be maintained at high frequencies. Addition of the $P2Y_1$ agonist MRS2365 10 μM during the purinergic rundown did not cause any hyperpolarization. Protein kinase C (PKC), a putative $P2Y_1$ desensitizator, was able to reduce the amplitude of the IJPf when activated, but the rundown was not modified by PKC inhibitors. Frequencies higher than 0.60 ± 0.15 Hz were needed to evoke a sustained nitrergic hyperpolarization that progressively increased reaching $IJPs_{\infty} = -13 \pm 0.4$ mV at high frequencies and leading to a sustained inhibition of spontaneous motility.

Conclusion: Changes in frequency of stimulation possibly mimicking neuronal firing will post-junctionally determine purinergic vs. nitrergic responses underlying different functional roles. NO will be responsible for sustained relaxations needed in physiological processes such as storage, while purinergic neurotransmission evoking sharp transient relaxations will be dominant in processes such as propulsion.

Keywords enteric neurones, inhibitory junction potential, propulsion, relaxation, smooth muscle, storage.

The main functions of the colon are (i) absorption of water and electrolytes, (ii) fermentation by bacteria of cellulose-based material, (iii) formation and storage of solid faeces until defecation and (iv) propulsion of faeces to the rectum. All of these physiological processes require different mechanisms of contraction and relaxation. To accomplish the varied purposes of each process, the type of relaxation needed is different. Correspondingly, neural-mediated relaxation in the human colon is due to the release of several

 doi: 10.1111/apha.12408

neurotransmitters such as ATP (Jimenez *et al.* 2014) and nitric oxide (NO) (Keef *et al.* 1993, Lecci *et al.* 2002, Sanger *et al.* 2013) from enteric inhibitory neurones that cause hyperpolarizations of the membrane potential of smooth muscle cells (SMC) termed inhibitory junction potentials (IJP).

Cotransmission was first proposed by Burnstock (1976) and refers to the co-release of two or more neurotransmitters from a single neurone (Burnstock 1976). IJP in the colon typically display a first fast purinergic component (IJPf) (Burnstock *et al.* 1970, Mutafova-Yambolieva *et al.* 2007, Durnin *et al.* 2012) followed by a slow nitrergic hyperpolarization (IJPs) (Bult *et al.* 1990, Crist *et al.* 1992, Boeckxstaens *et al.* 1993, He & Goyal 1993, Keef *et al.* 1993, Gallego *et al.* 2008). Various studies suggest that the purine and NO are released from the same nerve terminal (Chaudhury *et al.* 2012), and therefore, each physiological process may require different patterns of neuronal firing to evoke different relaxation motor patterns.

There is now general consensus that the $P2Y_1$ receptor is the one responsible for purinergic neurotransmission in the whole gastrointestinal tract (King 2012, Goyal *et al.* 2013) including the human small intestine (Gallego *et al.* 2014) and human colon (Gallego *et al.* 2006). Even though the underlying mechanism for purinergic hyperpolarization remains unsettled (Zizzo *et al.* 2006, MacMillan *et al.* 2012), it has been proposed to involve IP3 and diacylglycerol (DAG) production by phospholipase C-β (PLC-β). IP3 evokes Ca^{2+} release from the sarcoplasmic reticulum (Hu *et al.* 2003, Gao *et al.* 2006) leading to the opening of small conductance calcium-activated potassium channels ($SK_{(Ca)}$) (Vogalis & Goyal 1997) and consequently reducing the probability of opening of L-type Ca^{2+} channels in the SMC. This mechanism has been also recently identified in PDGRFα+ interstitial cells that might contribute to nerve-mediated purinergic neuromuscular transmission (Kurahashi *et al.* 2011, Baker *et al.* 2013). The increase in the concentration of cytosolic calcium and/or DAG activates protein kinase C (PKC), a kinase that has been reported to be responsible for $P2Y_1$ desensitization in platelets (Hardy *et al.* 2005) and endothelial cells (Rodriguez-Rodriguez *et al.* 2009). In the colon, when two consecutive IJPf are elicited close together, the second response amplitude is reduced and this phenomenon is known as rundown (King 1994, Matsuyama *et al.* 2003, Gallego *et al.* 2008). This fact suggests that purinergic hyperpolarizations cannot be maintained over time, evoking transient relaxations (Gallego *et al.* 2008).

Nitric oxide is produced by the neuronal NO synthase (nNOS) and diffuses through the plasma membrane. NO binding to guanylyl cyclase (GCα and β) produces cGMP, generating a cascade of phosphorylations due to the activation of a cGMP-dependent protein kinase (PKG). The ionic basis for nitrergic hyperpolarization has not been fully elucidated, being the opening of K^+ channels or closing of Cl^- conductance channels the main options under discussion (He & Goyal 2012). NO requires high frequencies of electrical field stimulation (EFS) to be released and causes a sustained hyperpolarization and relaxation (Gallego *et al.* 2008).

The aim of this work was to study the dynamics of purinergic and nitrergic cotransmission to identify possible different functional roles for each inhibitory pathway in the human colon. Determining the parameters of EFS possibly mimicking neurone firing that lead to the different relaxation patterns will help improve the testing of inhibitory neurotransmission in neuromuscular diseases and consequently their treatment (Jimenez *et al.* 2014).

Materials and methods

Ethical approval

The patients of this study provided written, informed consent, and the experimental procedure was approved by the Ethics Committee of the Hospital of Mataró (Barcelona, Spain).

Tissue preparation

Samples of sigmoid colon were obtained from patients (aged 41–89 year) during colon resections for neoplasm ($N = 36$, 19 men and 17 women). Colon segments from macroscopically normal regions were collected and transported to the laboratory in cold saline buffer. The tissue was placed in Krebs solution on a dissection dish and the mucosal layer was removed. The composition of the Krebs solution was as follows (in mM): 10.10 glucose, 115.48 NaCl, 21.90 $NaHCO_3$, 4.61 KCl, 1.14 NaH_2PO_4, 2.50 $CaCl_2$ and 1.16 $MgSO_4$ bubbled with a mixture of 5% CO_2:95% O_2 (pH 7.4).

Solutions and drugs

The following drugs were used: nifedipine, *N*ω-nitro-L-arginine (L-NNA), phentolamine, atropine sulphate, propranolol (Sigma Chemicals, St. Louis, MO, USA), sodium nitroprusside (NaNP) (Research Biochemicals International, Natick, MA, USA), MRS2500, MRS2365, Gö6983, GF109203X and PDBu (Tocris, Bristol, UK). Stock solutions were made by dissolving drugs in distilled water except for nifedipine which was dissolved in ethanol (96%) (<0.001% v/v), PKC

  doi: 10.1111/apha.12408

inhibitors (Gö6983, GF109203X) and PKC activator PDBu, dissolved in DMSO (<0.001% v/v) and L-NNA, which was dissolved in Krebs solution by sonication.

Electrophysiological experiments

Muscle strips were dissected parallel to the circular muscle and pinned, in a cross-sectioned slab, in a Sylgard-coated recording chamber continuously perfused with Krebs solution at 37 ± 1 °C. Phentolamine, atropine and propranolol at 1 μM were added to create non-adrenergic non-cholinergic (NANC) conditions. Preparations were allowed to equilibrate for approx. 1 h before experiments were undertaken. To record membrane potential responses to EFS and drugs, circular muscle cells were impaled with single sharp glass microelectrodes filled with 3 M KCl and with a tip resistance of 40–60 MΩ. Membrane potential was measured using a standard electrometer Duo773 (WPI, Sarasota, FL, USA). Tracings were displayed on an oscilloscope 4026 (Racal-Dana Ltd., Windsor, UK) and simultaneously digitalized (100 Hz) with PowerLab 4/30 system and CHart 5 software for Windows (both from ADInstruments, Castle Hill, NSW, Australia). Intramuscular nerve terminals were stimulated using a pair of silver chloride electrodes, one on each side of the preparation. To obtain stable impalements, the tissue was perfused with nifedipine (1 μM) to abolish mechanical activity.

Mechanical experiments

Circularly oriented muscle strips were examined in a 10 mL organ bath filled with Krebs solution at 37 ± 1 °C and NANC conditions. An isometric force transducer (Harvard VF-1) connected to an amplifier was used to record the mechanical activity. Data were digitalized (25 Hz) using DATAWIN1 software (Panlab, Barcelona, Spain) coupled to an ISC-16 analog-to-digital card installed in a PC. A tension of 4 g was applied to the colonic strips that were allowed to equilibrate for 1 h. After this period, strips displayed spontaneous contractions with a frequency of 2.89 ± 0.13 contractions per minute and a mean amplitude of 2.79 ± 0.80 g. Protocols of EFS consisted in 2 min of 50 V pulses of 0.4 ms of duration and at frequencies of 0.2, 0.6, 1, 2 and 5 Hz. These frequencies were established after performing electrophysiological experiments and are representative of different stages of the cotransmission process (see Results).

Data analysis and statistics

Electrophysiology. Nonlinear regression was used to fit experimental data (X = voltage or frequency of EFS vs. Y = amplitude of electrophysiological responses) to mathematical equations. Linear regression was used to test proportionality between IJPf and hyperpolarization induced with 5 Hz stimulation. A Student's unpaired t-test was used to compare the differences in the IJPf decay between control and L-NNA incubated tissue and between frequency increasing and isolated frequency trains of stimulation. Voltage-increasing trains in control and L-NNA-incubated tissue and agonist-induced hyperpolarizations on resting membrane potential (RMP) and during EFS were compared using an ANOVA test followed by a Bonferroni's multiple comparison test. Purinergic decay before and after PKC inhibitors incubation was compared using a one-way ANOVA. PDBu effect on the IJPf amplitude was tested using a Student's paired t-test.

Mechanical studies. The response to EFS was measured in intervals of 30 s and represented as a percentage of the basal AUC of contractions (100%) to normalize mechanical data. The response in each time interval was compared to the control using a one-way ANOVA followed by a Bonferroni *post hoc* test.

Statistical analysis was performed with GRAPHPAD PRISM 5 for Windows (GraphPad Software, San Diego, CA, USA). Data were considered significant when $P < 0.05$. N values represent the number of strips from different patients.

Results

Effect of voltage on purinergic responses

Electrophysiology. Single pulses (0.4 ms of duration) of EFS in NANC conditions in the human sigmoid colon elicited a purely purinergic IJPf, sensitive to the $P2Y_1$ antagonist MRS2500 (Gallego *et al.* 2011). Increasing voltages of EFS (8, 12, 16, 20, 24, 28, 32 and 40 V) caused an increase of the IJPf amplitude (Fig. 1a) fitting a sigmoid voltage–response curve (Fig. 1b) that was not modified by incubation with the NOS inhibitor L-NNA at 1 mM (two-way ANOVA, $P = 0.32$) (Fig. 1b). Experimental values are represented in Table 1.

Effect of frequency on the cotransmission process

Electrical field stimulation at a supramaximal voltage and increasing frequencies of stimulation (from 0.1 to 10 Hz) elicited a complex response consisting of sharp hyperpolarizations at low frequencies partially superimposed to a more sustained hyperpolarization at high frequencies (Fig. 2a). The amplitude of the sharp hyperpolarizations became smaller with incre-

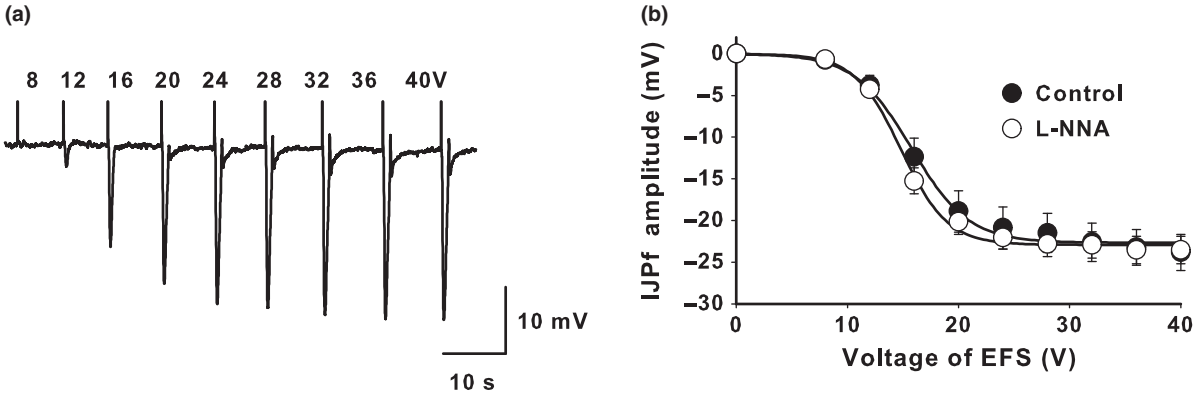

Figure 1 Purinergic response to voltage. (a) Microelectrode recording of single pulses at increasing voltages of stimulation. The IJPf increases its amplitude with voltage of electrical field stimulation. (b) Experimental data are fitted to a sigmoid voltage–response curve in control tissue (black dots, D.F. = 187; $R^2 = 0.55$; $N = 19$) and L-NNA 1 mM incubated tissue (white dots, D.F. = 187; $R^2 = 0.74$; $N = 19$). IJP, inhibitory junction potentials; L-NNA, *N*ω-nitro-L-arginine.

Table 1 Purinergic response to voltage ($N = 19$). Equation that represents purinergic response to voltage and experimental values of the purinergic voltage–response curve

$$\mathrm{IJPf}_V(\mathrm{mV}) = \mathrm{IJPf}_{\mathrm{MAX}} - \frac{\mathrm{IJPf}_{\mathrm{MAX}}}{1 + 10^{(Vf50 - V)\times \mathrm{Hillslope}(f)}}$$

	Value	Description
$\mathrm{IJPf}_{\mathrm{MAX}}$	−27.6 ± 1.6 mV	Maximum IJPf amplitude obtained with a single pulse
*Vf*50	15.8 ± 0.8 V	Voltage of electrical field stimulation at which the IJPf amplitude is half of the $\mathrm{IJPf}_{\mathrm{MAX}}$
Hill slope(*f*)	0.17 ± 0.04	Hill slope (steepness) of the fast component voltage–response curve

IJP, inhibitory junction potentials.

asing frequencies of stimulation, and concurrently, a progressively increasing long-lasting hyperpolarization was recorded at high frequencies (Fig. 2a,b).

Effect of frequency on isolated purinergic and nitrergic responses

To isolate the purinergic and nitrergic contribution to the cotransmission process, we performed the following experiments blocking the nitrergic component with L-NNA 1 mM and the purinergic component with MRS2500 1 μM. No response was recorded when the tissue was incubated with both MRS2500 and L-NNA (Fig. 3e,h), demonstrating that NANC neurotransmission at frequencies from 0.1 to 10 Hz is exclusively purinergic and nitrergic. Experimental values of the parameters from each electrophysiological response are represented in Table 2.

Purinergic neurotransmission decay with frequency

Electrophysiology. The purinergic IJPf decayed with frequency increase leaving a residual purinergic hyperpolarization at high frequencies of stimulation (IJPf_∞) (Fig. 3a) and fitting an exponential decay curve (Fig. 3b). The same attenuation of the IJPf amplitude occurred when isolated trains of fixed frequencies (0.1, 0.2, 0.3, 0.5, 0.7, 1, 3 and 5 Hz during 60 s, Fig. 5a,d,g) were applied (Fig. 4b). After a first fast hyperpolarization, the amplitude of the IJPf diminished to a level that was maintained for the remaining 60 s (steady state) (Fig. 4a,c). We evaluated the time needed to recover the first purinergic response after different frequencies of EFS eliciting a single stimulus every 30 s following each isolated train of stimulation (Fig. 4c). Data (frequency vs. time to recover 90% of the original IJPf amplitude) fitted an exponential function that saturates at 100.2 ± 12.2 s with frequencies of stimulation higher than 1 Hz (Fig. 4d).

Mechanical studies. Purinergic neurotransmission at 0.2 Hz was able to cause phasic relaxations of the tissue (Fig. 5b). Consequently, purinergic release was not able to reduce the basal AUC of contractions in a sustained manner. The inhibitory effect on the AUC due to phasic relaxations was camouflaged by the fact

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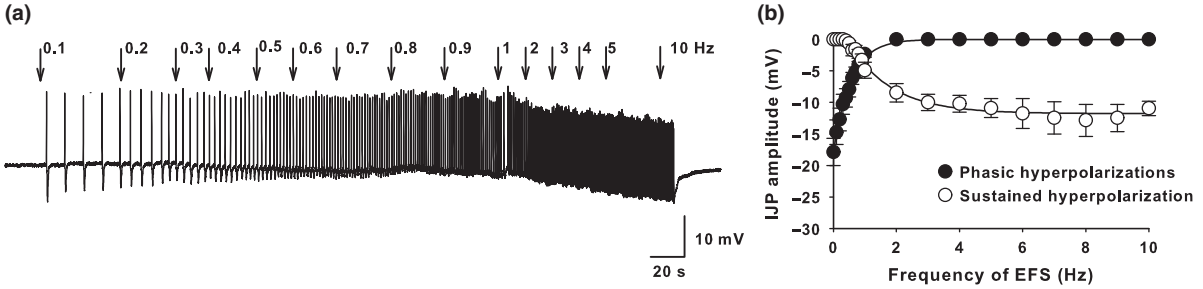

Figure 2 Cotransmission response to increasing frequencies of stimulation. (a) Microelectrode recording of a frequency-increasing train of electrical field stimulation (from 0.1 to 10 Hz). (b) Phasic hyperpolarizations amplitude fitted to an exponential decay (black dots, D.F. = 146; $R^2 = 0.8$, $N = 8$) and the sustained hyperpolarization fitted to a exponential increase curve (white dots, D.F. = 144; $R^2 = 0.7$, $N = 8$).

that after each phasic relaxation, there was an off-contraction and when this off-contraction was taking place, the next phasic relaxation appeared giving an apparent increase in the tone. A similar effect took place at 0.6 Hz. At 1, 2 and 5 Hz, purinergic neurotransmission was able to cause cessation of spontaneous activity during the first seconds of EFS, but contractions tended to recover during the rest of the stimulus (Fig. 5e,h). This effect on motility was in accordance with the purinergic electrophysiological response. The first IJPf was able to inhibit phasic contractions but afterwards, due to the rundown, the purinergic response is reduced and myogenic contractions recover (Fig. 5d,g).

Nitrergic neurotransmission increase with frequency

Electrophysiology. Frequencies higher than 0.60 ± 0.15 Hz (F_0) were needed to evoke a progressive and sustained nitrergic hyperpolarization that reached a maximum response at 5 Hz ($IJPs_\infty$) fitting an exponential increase curve (Fig. 3c,d). At isolated frequencies of 0.2, 0.6, 1, 2 and 5 Hz, nitrergic responses were not different from the ones obtained in the frequency-increasing protocols. Therefore, at 0.2 Hz, no nitrergic responses were recorded (Fig. 6a), while at high frequencies nitrergic neurotransmission caused a sustained hyperpolarization (Fig. 6d,g).

Mechanical studies. According to the results obtained in the electrophysiological studies, EFS did not relax the tissue at 0.2 Hz (Fig. 6b) and started to slightly inhibit contractions at 0.6 Hz. In contrast to purinergic neurotransmission, at high frequencies (1, 2 and 5 Hz), EFS was able to inhibit contractions through the entire stimulus (Fig. 6e,h).

Representation of the cotransmission process taking into account the increase of inhibitory responses with voltage and the opposite behaviour of each neurotransmitter with frequency is shown in Figure 7 and graphically represents the following modelling equations:

Purinergic response

$$\mathrm{IJPf}_{F,V}(\mathrm{mV}) = ((\mathrm{IJPf}_{\mathrm{MAX}} - \mathrm{IJPf}_\infty) \times e^{-k(f)F}) + \mathrm{IJPf}_\infty \times \left(1 - \frac{1}{1 + 10^{(Vf50-V)\times \mathrm{Hillslope}(f)}}\right)$$

Nitrergic response

If F (Hz) < F_0 then $\mathrm{IJPs}_{F,V} = 0$ mV
If F (Hz) > F_0 then:

$$\mathrm{IJPs}_{F,V}(\mathrm{mV}) = \mathrm{IJPs}_\infty(1 - e^{-k(s)(F-F_0)}) \times \left(1 - \frac{1}{1 + 10^{(Vs50-V)\times \mathrm{Hillslope}(s)}}\right)$$

Evidence supporting cotransmission

If purines and NO have a common neuronal release, purinergic and nitrergic responses should have a linear relationship. To test this possible proportionality, we analysed the purinergic and nitrergic responses obtained at 5 Hz of stimulation during 20 s with 20 impalements of 20 different patients. The amplitudes of the nitrergic responses were plotted against the purinergic IJPf. The obtained correlation coefficient was of 0.66, and slope was significantly different from zero ($P < 0.0001$) (Fig. 8).

Purinergic rundown is due to post-junctional desensitization

To achieve a possible explanation for the purinergic rundown, we performed a protocol that consisted of

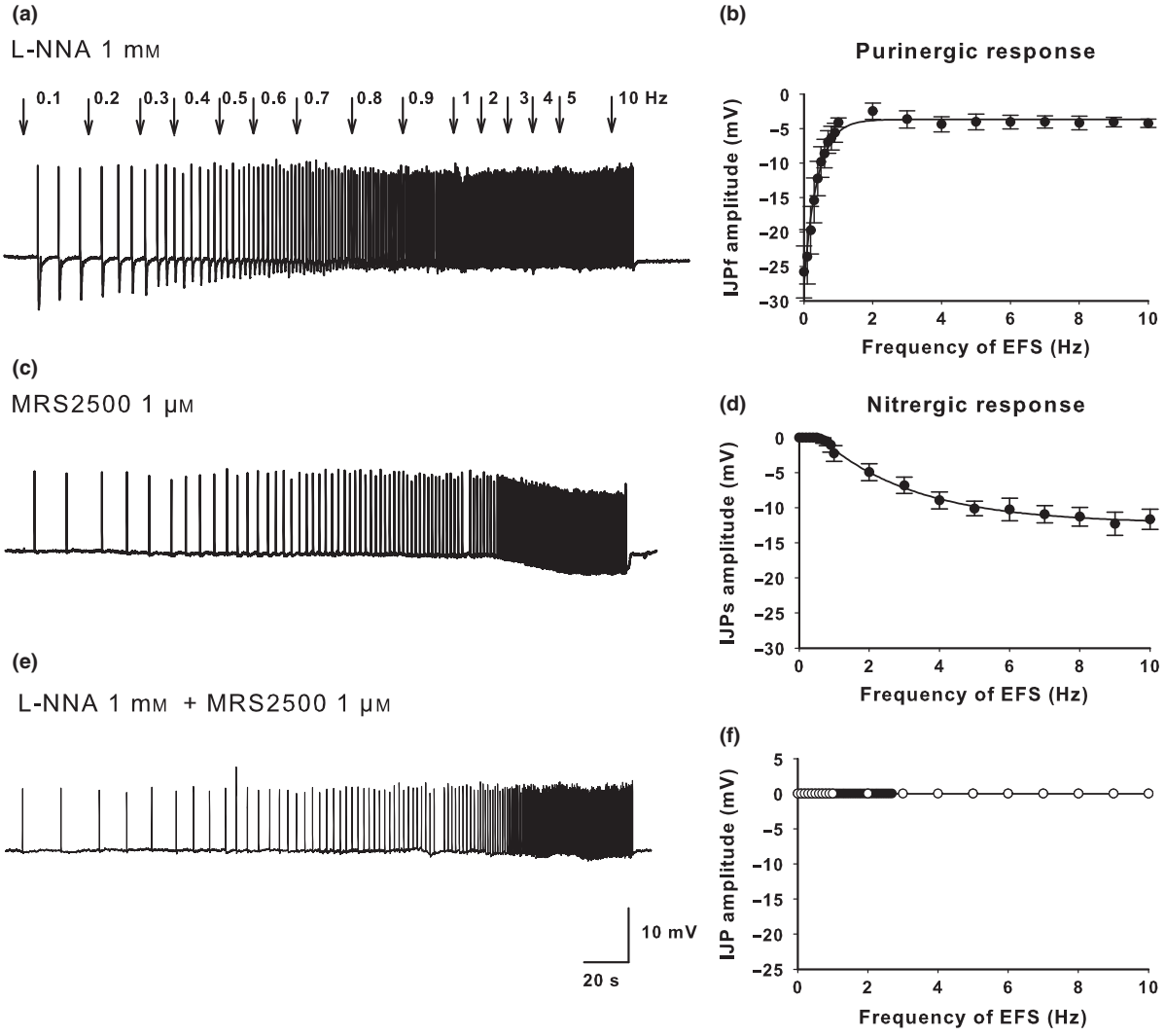

Figure 3 Purinergic and nitrergic responses to frequency increase. (a) Microelectrode recording of a frequency-increasing train of electrical field stimulation in an L-NNA 1 mM incubated preparation, in MRS2500 1 μM incubated tissue (c) and in L-NNA 1 mM and MRS2500 1 μM incubated tissue (e). (b) Purinergic response fitted to an exponential decay curve (D.F = 117; $R^2 = 0.7$; $N = 6$). (d) Nitrergic response fitted to an exponential increase curve (D.F = 97; $R^2 = 0.8$; $N = 6$). (f) No response was recorded when the tissue was incubated with both L-NNA 1 mM and MRS2500 1 μM. L-NNA, *N*ω-nitro-L-arginine.

adding the $P2Y_1$ agonist MRS2365 at 10 μM during the purinergic rundown in L-NNA 1 mM incubated tissue (Fig. 9b). Previously, to prove that MRS2365 10 μM hyperpolarized the preparation membrane potential, it was added during RMP and a hyperpolarization of about 10 mV was observed (Fig. 9a). When MRS2365 was added during the purinergic rundown, no hyperpolarization occurred. To show the SMC membrane potential capacity to hyperpolarize during the rundown, we performed the same protocol with NaNP 10 μM (Fig. 9a,b), observing a similar hyperpolarization after both additions (Fig. 9c).

As the prior result suggested that the rundown was due to post-junctional mechanisms and $P2Y_1$ phosphorylation by PKC has been described as a mechanism of desensitization of this receptor, we incubated the tissue (20 min) with the non-isoform-selective PKC inhibitors Gö6983 and GF109203X 10 μM. One-way ANOVA test showed no significant effect of PKC inhibitors on the decay of the purinergic response in frequency-increasing stimulation ($P = 0.22$) (Fig. 10a). However, tissue incubation with the PKC activator PDBu was able to reduce the amplitude of the IJPf (paired *t*-test $P < 0.05$), suggesting that PKC can desensitize the $P2Y_1$ receptor (Fig. 10b,c).

Discussion

The aim of the present study was to characterize the dynamics of the nerve-mediated electrophysiological

Table 2 Purinergic ($N = 6$) and nitrergic response ($N = 6$) to frequency. Equations that represent purinergic and nitrergic responses to frequency and experimental values of the purinergic and nitrergic frequency–response exponential curves

Purinergic response:

$IJPf_F(mV) = (IJPf_{MAX} - IJPf_\infty) \times e^{-k(f)F} + IJPf_\infty$

Nitrergic response:

If $F(Hz) < F_0$ then $IJPs_F = 0$ mV

If $F(Hz) > F_0$ then $IJPs_F(mV) = (IJPs_\infty) + 1 - e^{-k(s)(F-F_0)}$

Purinergic response	Nitrergic response	Description
$IJPf_{MAX} = -27.6 \pm 1.6$ mV	None*	Maximum IJP amplitude obtained with a single pulse
F_0 = None*	0.60 ± 0.15 Hz	F_0 is the frequency of EFS below which no response is recorded
$k(f) = 2.6 \pm 0.3$ s	$k(s) = 0.3 \pm 0.1$ s	Time constant. $1/k$ is the frequency at which 63.2% of the response is attenuated (purinergic)/increased (nitrergic)
$IJPf_\infty = -3.7 \pm 0.6$ mV	$IJPs_\infty = -13.3 \pm 0.4$ mV	Amplitude of the response obtained at high frequencies of stimulation

IJP, inhibitory junction potentials; EFS, electrical field stimulation.

*Single pulses never elicited nitrergic response unless trains of frequencies higher than 0.60 ± 0.15 Hz were applied, while purinergic responses were present at all frequencies of EFS.

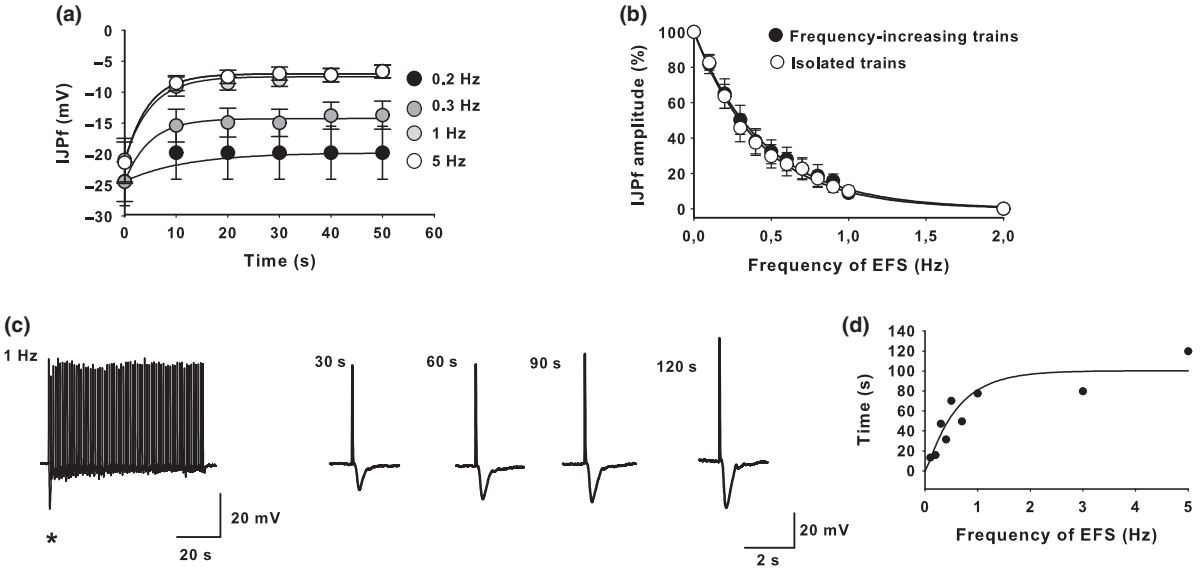

Figure 4 Purinergic response in isolated trains of electrical field stimulation (EFS) and recovery of the original IJPf amplitude after the rundown. (a) Graph representing the decay of the IJPf during EFS at different frequencies of stimulation. (b) Decay (k (s)) of the purinergic IJP with frequency in frequency-increasing trains of stimulation (D.F = 72; $R^2 = 0.6$; $N = 5$) and in isolated trains of fixed frequencies (D.F = 47; $R^2 = 0.7$; $N = 5$) was not significantly different (Student's unpaired t-test, $P = 0.62$). (c) Microelectrode recording of an isolated 60-s train of stimulation at a frequency of 1 Hz and recordings of the IJPf obtained with single pulses every 30 s until 120 s after the stimulation train. (d) Black dots represent the mean of the time needed after each frequency of stimulation to recover 90% of the first IJPf (*) and are fitted to an exponential function (D.F. = 5; $R^2 = 0.85$; $N = 5$). IJP, inhibitory junction potentials.

inhibitory responses in the human colon using different frequencies of EFS and to correlate the different electrophysiological responses with colonic relaxation. The relative contribution of each neurotransmitter to hyperpolarization and relaxation was characterized using MRS2500 1 μM and L-NNA 1 mM to isolate the nitrergic and purinergic component respectively. This experimental approach has been recently performed in rat colon where we studied how the purine-NO cotransmission process interacts with pacemaker function (Mane *et al.* 2014). Previous studies have characterized nerve–muscle interaction in the human gastrointestinal tract and have demonstrated that both in the human small and large intestine, the IJP elicited with single pulses/short trains of EFS is purinergic (Xue *et al.* 1999, Gallego *et al.* 2006, 2011, 2014)

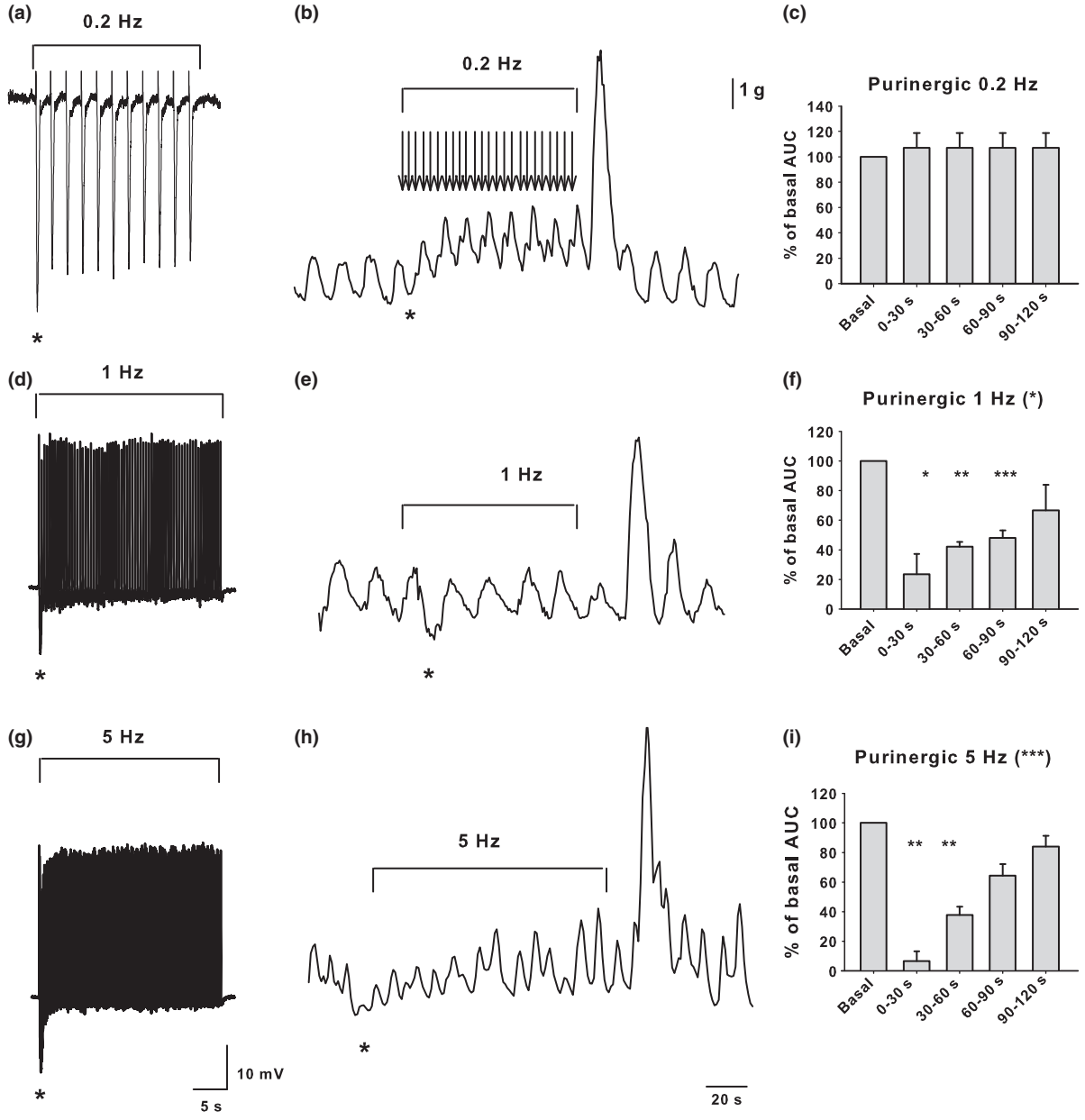

Figure 5 Purinergic IJP and relaxation in response to isolated frequencies of electrical field stimulation (EFS). (a) Microelectrode recordings of isolated trains of EFS at a frequency of 0.2 Hz, 1 Hz (d) and 5 Hz (g). A first IJPf is displayed at the beginning of the stimulus (*), but it attenuates rapidly. (b) Mechanical recordings of the effect of purinergic neurotransmission on spontaneous contractions at a frequency of 0.2 Hz, 1 Hz (e) and 5 Hz (h). In (b), arrows represent single stimuli. Notice how, at 0.2 Hz, when there is a pulse, purinergic release causes a phasic relaxation interrupting the contraction. This is rapidly followed by an off-contraction that again is interrupted by the next stimulus. At 1 and 5 Hz, notice how only at the beginning of the stimulus, purinergic neurotransmission is able to cause a transient inhibition of contractions (*). Afterwards, mechanical activity is restored tending to reach the basal AUC. (c) Histograms representing the % of the basal AUC during the 120 s in 30-s intervals of EFS at 0.2 Hz, 1 Hz *($P < 0.05$) (f) and 5 Hz ***($P < 0.001$) (i). IJP, inhibitory junction potentials.

and that short time intervals between single pulses elicit consecutive IJPf that rundown (Gallego *et al.* 2008). To evoke nitrergic responses, however, high frequencies of EFS are needed (Keef *et al.* 1993).

In the present study, we demonstrate that the process of inhibitory cotransmission is highly dependent on the frequency of nerve stimulation. One or another neurotransmitter is transduced within the SMC depending on the frequency of EFS used. This behaviour of cotransmission is known as 'fast and slow cotransmitters' (Burnstock 2004): the fast cotransmitter, in our case, the purine, is released with

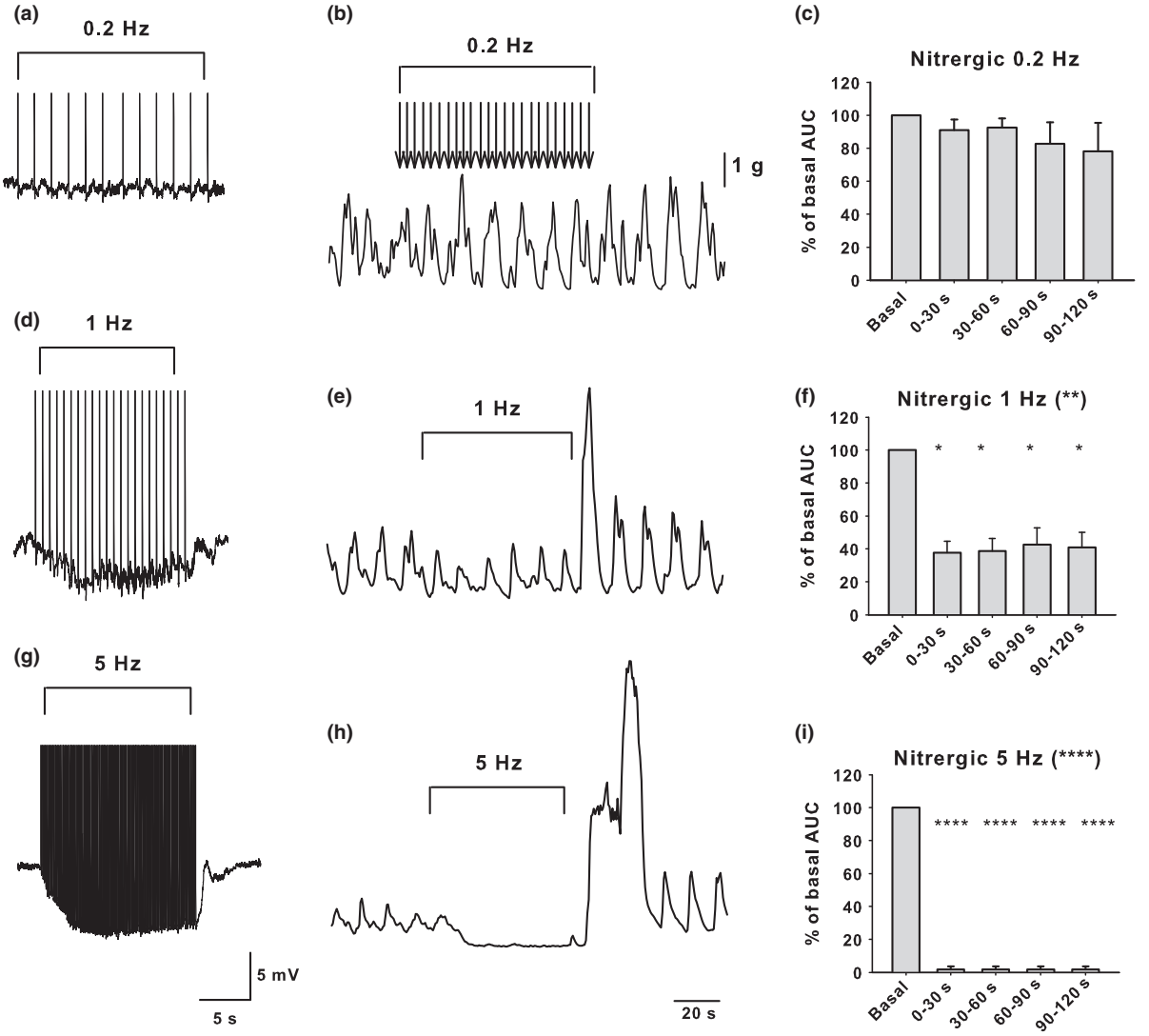

Figure 6 Nitrergic IJP and relaxation in response to isolated frequencies of electrical field stimulation (EFS). (a) Microelectrode recordings of isolated trains of EFS at a frequency of 0.2 Hz, 1 Hz (d) and 5 Hz (g). No nitrergic response is recorded at 0.2 Hz, but at 1 and 5 Hz, NO causes a sustained hyperpolarization. (b) Mechanical recordings of the effect of nitrergic neurotransmission on spontaneous contractions at a frequency of 0.2 Hz, 1 Hz (e) and 5 Hz (h). In (b), arrows represent single stimuli. Notice how no nitrergic effect on mechanical activity is observed at 0.2 Hz. At 1 and 5 Hz, NO can cause a sustained relaxation through all the EFS. (c) Histograms representing the % of the basal AUC during the 120 s in 30-s intervals of EFS at 0.2 Hz, 1 Hz **($P < 0.01$)(f) and 5 Hz ****($P < 0.0001$) (i). IJP, inhibitory junction potentials; NO, nitric oxide.

single pre-synaptic action potentials, while the slow cotransmitter, in our case NO, requires high frequencies of neuronal stimulation to be released. Therefore, at low frequencies of EFS, the predominant post-junctional response is purinergic. At high frequencies of EFS, however, there is a frequency-dependent attenuation of purinergic responses leaving a residual purinergic response of 3–4 mV. The IJPf rundown transiently overlaps with the frequency-dependent summation in nitrergic response that reaches 13–14 mV.

The reason for the purinergic rundown could be pre-junctional (neurotransmitter depletion or pre-junctional inhibition) or post-junctional (desensitization of the post-junctional pathway). If decrease of purinergic nerve release was the cause of the rundown, then the SMC should hyperpolarize during EFS after external addition of the $P2Y_1$ agonist MRS2365, but this was not the case. These results suggest that there are no $P2Y_1$ receptors available to transduce the purinergic signal, and therefore, exogenous addition of the $P2Y_1$ agonist cannot further hyperpolarize the SMC. Moreover, recent studies that propose ß-NAD and ADP-R as the purinergic neurotransmitters in the gastrointestinal tract, including the human colon (Hwang *et al.* 2011),

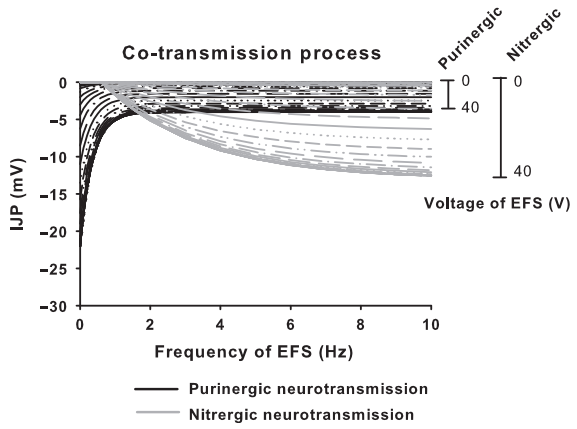

Figure 7 Representation of the cotransmission process (values of Tables 1 and 2). Increases of voltage of electrical field stimulation (EFS) increase neurotransmission and inhibitory responses (right *Y* axis for 10 Hz). Increases of frequency of EFS (*X* axis) increase nitrergic (dark grey lines) but attenuate purinergic responses (black lines).

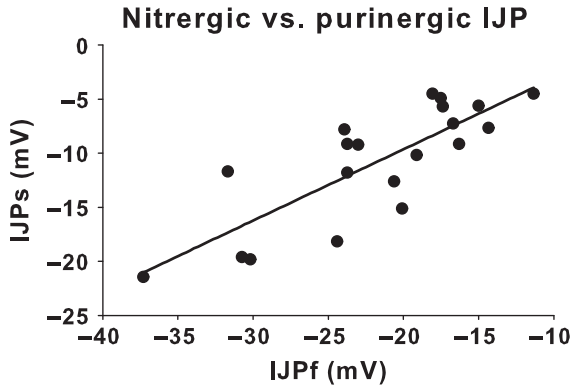

Figure 8 Linear relationship between purinergic and nitrergic neurotransmission. Nitrergic hyperpolarization plotted against purinergic response obtained at 5 Hz of 20 impalements of different samples. Impalements that exhibit high-amplitude IJPf also show high-amplitude IJPs, while impalements with low-amplitude IJPf have low-amplitude IJPs. IJP, inhibitory junction potentials.

have shown that nerve stimulation at 4 and 16 Hz elicits a frequency-dependent increase in purine release both in murine and human colonic tissue (Mutafova-Yambolieva *et al.* 2007, Hwang *et al.* 2011). According to our results, at 4 and 16 Hz of stimulation, the maximum rundown is already reached, and therefore, these data are also inconsistent with the pre-junctional hypothesis because, apparently, enteric neurones release more neurotransmitter at higher frequencies (Hwang *et al.* 2011, Durnin *et al.* 2012). Interestingly, we were able to measure the recovery of the rundown and found a parallelism between the frequency dependency of the rundown and its recovery. Stimulation at frequencies higher than 1 Hz probably saturates post-junctional receptors occupancy as, above this frequency, time to recover 90% of the original IJP is the same for all frequencies (about 100 s). These data are also consistent with the post-junctional hypothesis. Furthermore, the purine metabolite adenosine does not modify the time course of the IJP recovering which is not consistent with a mechanism of pre-synaptic inhibition (Gallego *et al.* 2008).

The increase in the concentration of cytosolic calcium and/or DAG activates PKC, a kinase that has been reported to be responsible for $P2Y_1$ desensitization in platelets (Hardy *et al.* 2005) and endothelial cells (Rodriguez-Rodriguez *et al.* 2009). PKC inhibitors in our case did not modify the purinergic rundown. However, the PKC activator PDBu was able to reduce the amplitude of the IJPf, and therefore, PKC is probably able to desensitize the $P2Y_1$ receptor, but we were not able to associate this mechanism with the purinergic rundown.

Our data are consistent with a cotransmission process (Burnstock 1976), that is both neurotransmitters are released from the same neurone. Five Hertz pulses at supramaximal voltage elicit proportional purinergic and nitrergic IJP. If cotransmitters were released from different neurones then it was conceivable that some SMCs would receive more nitrergic innervations, while others would receive more purinergic inputs, and this was not the case. However, despite a possible common nerve origin, the cell responsible for transducing signals to the SMC is still under debate. It is possible that ICCs transduce nitrergic signals (Ward & Sanders 2001, Groneberg *et al.* 2011, Lies *et al.* 2014), while PDGRFα+ cells could be responsible for transduction of purinergic signals to SMCs (Kurahashi *et al.* 2011, Baker *et al.* 2013). Interestingly, PDGRFα+ cells have been recently described in the human colon (Kurahashi *et al.* 2012) and have the apparatus responsible for purinergic neurotransmission including the $P2Y_1$ receptor, SK channels and the intracellular pathway responsible for the hyperpolarization and the rundown.

Under high frequencies of stimulation, the predominant neurotransmitter causing smooth muscle relaxation is NO. GC/PKG1 (Groneberg *et al.* 2011, Klein *et al.* 2013, Lies *et al.* 2013) activation by NO results in a tonic relaxation of the muscle and, despite possible purine release, the signalling mechanism ($P2Y_1$/$SK_{(Ca)}$) is desensitized. The mechanical consequence of this phenomenon is that the relaxations achieved with purinergic neurotransmission are transient.

According to these data, it is reasonable to speculate that nerve–muscle interaction and the type of relaxation displayed by the organ will depend on the firing frequency of neurones. Nicotine-evoked $[Ca^{2+}]_i$

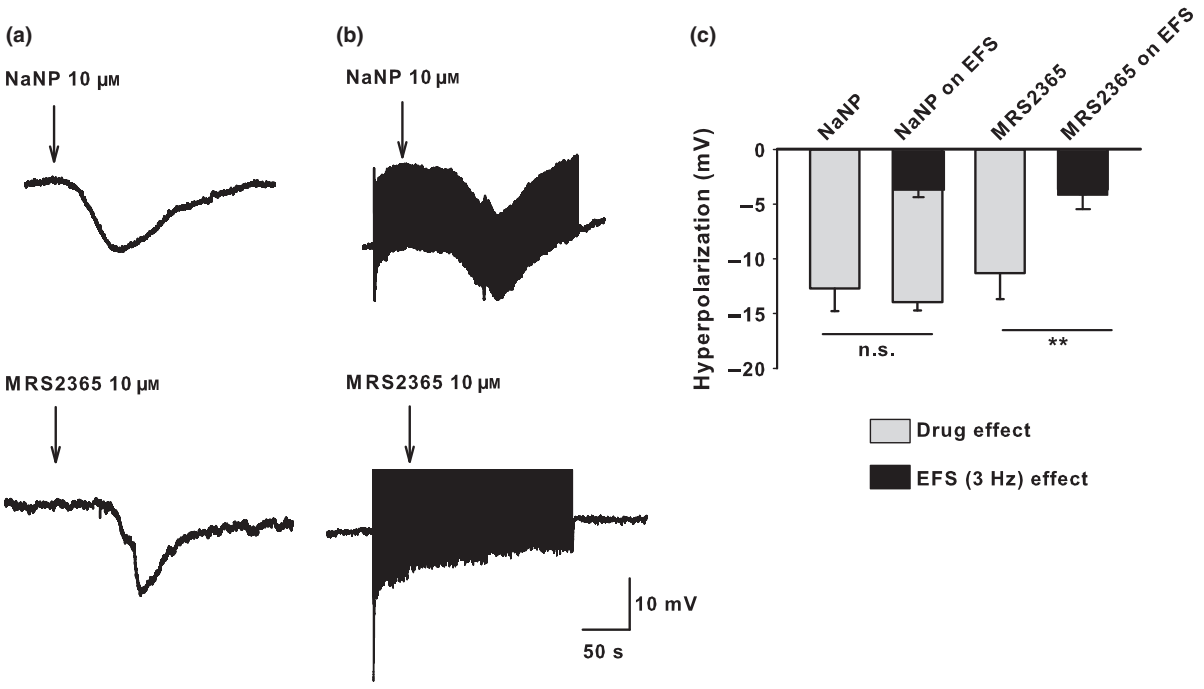

Figure 9 Purinergic rundown is due to post-junctional desensitization. (a) NaNP 10 μM (top) and MRS2365 10 μM (bottom) both hyperpolarized membrane potential of smooth muscle cells (SMCs) in resting membrane potential (RMP) conditions. (b) NaNP 10 μM (top) but not MRS2365 10 μM (bottom) hyperpolarized the membrane potential of SMC during the purinergic rundown. (c) Histogram showing the hyperpolarization observed with drug addition during RMP and during the purinergic rundown in grey. In black, purinergic hyperpolarization due to electrical field stimulation (EFS). ANOVA test followed by Bonferroni's multiple comparison test showed significant differences between the MRS2365 10 μM hyperpolarization observed with no EFS and the one observed when adding MRS2365 10 μM during EFS **($P < 0.01$) ($N = 4$).

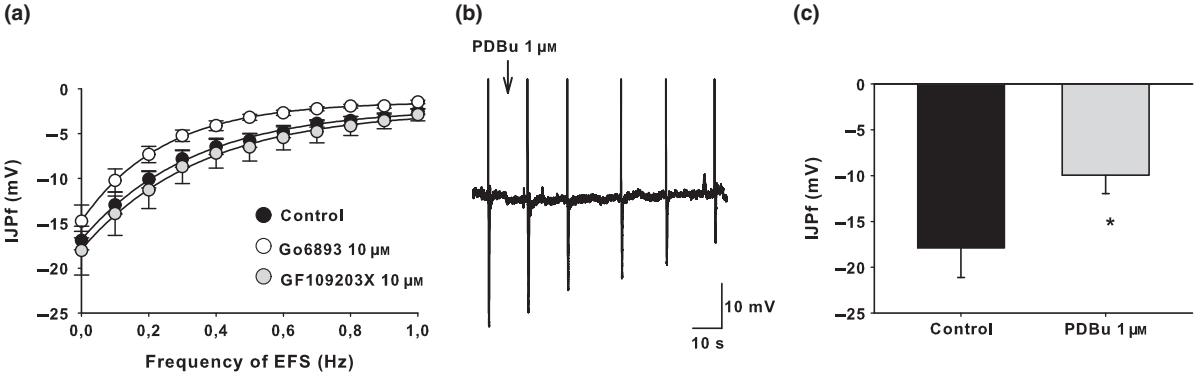

Figure 10 PKC is able to desensitize the $P2Y_1$ receptor, but this mechanism cannot be associated with the purinergic rundown. (a) PKC inhibitors Gö6983 ($N = 12$) and GF109203X ($N = 10$) at 10 μM did not modify the purinergic rundown. (b) Reduction of the IJPf amplitude at the supramaximal voltage during incubation with the PKC activator PDBu 1 μM. (c) Amplitude of the control supramaximal IJPf and the supramaximal IJPf after PDBu 1 μM incubation were significantly different *($P < 0.05$) ($N = 5$). IJP, inhibitory junction potentials; PKC, protein kinase C.

peaks associated with action potentials occur at a frequency of 7.3 ± 4.5 Hz in enteric neurones from the guinea-pig myenteric plexus and from the human submucous plexus (Michel *et al.* 2011), suggesting that the range of firing of enteric neurones is between 0 and 15 Hz. Similar results are obtained when enteric submucous neurones are stimulated with 5-HT_3 agonists (Buhner *et al.* 2009). This range of putative physiological firing frequencies is similar to the range of frequencies of EFS used in the present study.

Accordingly, we hypothesize that low firing frequencies of enteric inhibitory neurones (purinergic neurotransmission) are dominant when physiological processes that require transient relaxations such as propulsion are taking place, while inhibitory neurones will fire at high frequencies (NO) to elicit the sustained relaxations needed in processes such as storage. If this is true, it would be reasonable to speculate that purinergic neurotransmission is dominant in the descending and sigmoid colon (present study) where propulsion is the main function, while nitrergic neurotransmission is probably more present in the ascending and transverse colon, which have more of a 'storage' function. Future studies are needed to investigate these regional differences in the inhibitory cotransmission process. When referring to pathological implications, it is known that mediators (histamine, serotonin and proteases) obtained from supernatants of irritable bowel syndrome patients increase the frequency firing of submucosal neurones (Buhner *et al.* 2009). Consequently, if this also occurs in myenteric neurones, under certain pathological circumstances, the predominant neurotransmitter might change modifying colonic motility. In addition, purinergic neurotransmission implication in gastrointestinal motility diseases has not been studied. As the diagnosis of neuromuscular diseases of the gastrointestinal tract including Hirschsprung, achalasia and intestinal or colonic pseudo-obstruction is mainly based on anatomopathological findings (Knowles *et al.* 2010), our model can help establish the protocols to analyse both components of inhibitory neurotransmission in human gastrointestinal samples.

Conflict of interest

The authors state no conflict of interest.

This work has been funded by the following grant: BFU2009-11118 and 2011 CTP 00032. Noemí Mañé (AP2012-0897), Víctor Gil (AP2007-01583) and Míriam Martínez-Cutillas (AP2010-2224) are supported by the Ministerio de Ciencia e Innovación (Spain). Diana Gallego is supported by the Instituto de Salud Carlos III, Centro de Investigación Biomédica en red de enfermedades hepáticas y digestivas (CIBERehd).

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CHAPTER 3

Inverse gradient of nitrergic and purinergic inhibitory cotransmission in the mouse colon

Acta Physiol (Oxf), **216**, 120-131.

ACTA PHYSIOLOGICA

Acta Physiol 2016, **216,** 120–131

Inverse gradient of nitrergic and purinergic inhibitory cotransmission in the mouse colon

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Abstract

Aim: Gastrointestinal smooth muscle relaxation is accomplished by the neural corelease of ATP or a related purine and nitric oxide. Contractions are triggered by acetylcholine and tachykinins. The aim of this work was to study whether regional differences in neurotransmission could partially explain the varied physiological roles of each colonic area.

Methods: We used electrophysiological and myography techniques to evaluate purinergic (L-NNA 1 mM incubated tissue), nitrergic (MRS2500 0.3 μM incubated tissue) and cholinergic neurotransmission (L-NNA 1 mM and MRS2500 0.3 μM incubated tissue) in the proximal, mid and distal colon of CD1 mice (n = 42).

Results: Purinergic electrophysiological responses elicited by single pulses (28 V) were greater in the distal ($IJPf_{MAX} = -35.3 \pm 2.2$ mV), followed by the mid ($IJPf_{MAX} = -30.6 \pm 1.0$ mV) and proximal ($IJPf_{MAX} = -11.7 \pm 1.1$ mV) colon. In contrast, nitrergic responses decreased from the proximal colon ($IJPs_{MAX} = -11.4 \pm 1.1$ mV) to the mid ($IJPs_{MAX} = -9.1 \pm 0.4$ mV), followed by the distal colon ($IJPs_{MAX} = -1.8 \pm 0.3$ mV). A similar rank of order was observed in neural mediated inhibitory mechanical responses including electrical field stimulation-mediated responses and neural tone. ADPβs concentration–response curve was shifted to the left in the distal colon. In contrast, NaNP responses did not differ between regions. Cholinergic neurotransmission elicited contractions of a similar amplitude throughout the colon.

Conclusion: An inverse gradient of purinergic and nitrergic neurotransmission exists through the mouse colon. The proximal and mid colon have a predominant nitrergic neurotransmission probably due to the fact that their storage function requires sustained relaxations. The distal colon, in contrast, has mainly purinergic neurotransmission responsible for the phasic relaxations needed to propel dehydrated faeces.

Keywords colonic relaxation, nitric oxide, purines, regional differences.

Nerve-mediated smooth muscle contraction and relaxation are needed to accomplish several colonic motor functions such as absorption, mixing, storage and propulsion. Although excitatory enteric neurones corelease other transmitters such as tachykinins (Holzer & Holzer-Petsche 1997), acetylcholine (Ach) is believed to be functionally predominant in inducing contractions in the gut (Goyal & Hirano 1996,

 doi: 10.1111/apha.12599

Furness 2000). ATP (Burnstock *et al.* 1970) and nitric oxide (NO) (Bult *et al.* 1990) are the non-adrenergic non-cholinergic (NANC) inhibitory cotransmitters responsible for relaxation (Burnstock 1976, Chaudhury *et al.* 2012). Recently, we have reported that differences in the frequency of electrical field stimulation (EFS) probably mimicking neural firing (Michel *et al.* 2011) are responsible for determining the predominant inhibitory neuromuscular transmission pathway and consequently, the kind of relaxation achieved (Mane *et al.* 2014a,b). Specifically, purinergic neurotransmission is dominant when single pulses or short trains of EFS are elicited, evoking a fast inhibitory junction potential (IJPf) in smooth muscle cells (SMCs) through the activation of $P2Y_1$ receptors (Gallego *et al.* 2006, 2012, Grasa *et al.* 2009). The amplitude of the IJPf attenuates with prolonged stimuli at high frequencies of EFS, and therefore, purinergic neurotransmission is only able to cause transient relaxations of colonic muscles (Gallego *et al.* 2008, Mane *et al.* 2014a,b). In contrast, nitrergic neurotransmission is potentiated by prolonged high frequencies of EFS, which cause a sustained hyperpolarization or slow IJP (IJPs) in the SMC leading to long-lasting colonic relaxations (Gallego *et al.* 2008, Mane *et al.* 2014a,b).

The colon can be divided into two distinct regions on the basis of different embryological origins, innervation and blood supply: proximal and distal to the splenic flexure (Christensen 1991). These differences are also reflected by differences in the function of the proximal and distal colon. The proximal colon has a high reservoir capacity and is considered the primary site of storage for faeces (Ford *et al.* 1995). The delay in transit of faeces through the ascending colon serves to enhance the absorption of fluid excess. In contrast, the distal colon displays mainly phasic contractility (Hertz & Newton 1913, Bassotti *et al.* 1987, 1989, Dapoigny *et al.* 1988, Christensen 1991, Dinning *et al.* 2012, 2014, Spencer *et al.* 2012) to propel dehydrated faeces with intense peristaltic contractions (Ritchie 1968, Snipes *et al.* 1982).

The aim of the present work was to study cholinergic, purinergic and nitrergic neuromuscular transmission in different regions of the mouse colon to associate a different neurotransmission pattern to each colonic segment and, therefore, to establish a functional role for each neurotransmitter.

Material and methods

Ethical approval

All procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona.

Animals and tissue samples

Forty-eight CD1 female mice of 8–18 weeks old were purchased from Charles River. Animals were housed under controlled conditions: constant temperature (22 ± 2 °C) and humidity ($55 \pm 10\%$), 12-h : 12-h light : dark cycle and *ad libitum* access to water and food. Animals were killed by cervical dislocation. The colon was quickly removed (distally to the caecum and proximally to the rectum) and placed in carbogenated (95% O_2 and 5% CO_2) Krebs solution. The mesenteric fat was removed, and the colon was opened along the mesenteric border and pinned onto a Sylgard (Sigma Chemicals, St. Louis, MO, USA) base with the mucosa side up. We divided the colon in three segments: proximal, mid and distal colon. The proximal colon has a characteristic mucosa with folds oriented 45° from the longitudinal axis, the mid colon displays longitudinally oriented folds, and the distal colon mucosa is smooth. Mucosal and submucosal layers were gently removed. 3×5 mm muscle strips were cut in a circular direction.

Electrophysiological studies

Muscle strips were pinned in a Sylgard-coated chamber with the circular muscle layer facing upwards. The tissue was continuously perfused with carbogenated Krebs solution at 37 ± 1 °C and allowed to equilibrate for 1 h. Phentolamine, propranolol and atropine (all at 1 μM) were added to, respectively, block α- and β-adrenoceptors and muscarinic receptors and create NANC conditions. To obtain stable microelectrode impalements, nifedipine 1 μM was added to abolish mechanical activity. Circular SMCs were impaled using glass microelectrodes filled with KCl 3 M (30–60 MΩ of tip resistance). Membrane potential was measured by a standard Duo 773 electrometer (WPI, Sarasota, FL, USA). Tracings were displayed on an oscilloscope (Racal–Dana, Windsor, UK) and simultaneously digitalized (100 Hz) with a PowerLab 4/30 system and CHART 5 software for Windows (both from ADInstrument, Castle Hill, NSW, Australia). IJPs were elicited by EFS using two silver chloride plates placed 1.5 cm apart perpendicular to the longitudinal axis of the preparation. Different protocols of stimulation were performed: single pulses, 0.4 ms duration, at increasing voltages of EFS (8, 12, 16, 20, 24, 28, 32, 36, 40 V) and frequency trains using the supramaximal voltage and increasing frequencies of EFS (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5 Hz). The amplitude of the IJP (mV) was measured considering it as the difference between the maximal hyperpolarization and the resting membrane potential (RMP).

Mechanical studies

Muscle strips were mounted in a 10 mL organ bath containing carbogenated Krebs solution maintained at 37 ± 1 °C. A tension of 0.5 g was applied, and tissues were allowed to equilibrate for 1 h. After this period, strips displayed tetrodotoxin (TTX)-resistant spontaneous myogenic phasic activity (Domenech *et al.* 2011). Mechanical activity was measured using an isometric force transducer (UF-1 Harvard Apparatus, Holliston, MA, USA) connected to a computer through an amplifier. Data were digitalized (25 Hz) using DATA 2001 software (Panlab, Barcelona, Spain) coupled to an A/D converter installed in the computer. Cholinergic neurotransmission was studied in non-purinergic (MRS2500 0.3 μM) non-nitrergic (L-NNA 1 mM) (NNNP) conditions and in the presence of phentolamine and propranolol at 1 μM. The parameters of EFS used were pulses at a frequency of 30 Hz, 0.4 ms of duration and 1 s of total duration at voltages of 1, 5, 10, 15, 20 and 30 V. The release of inhibitory neurotransmitters was studied in NANC conditions using EFS at a frequency of 5 Hz and increasing voltages (1, 3, 5, 7, 10, 15, 20 V, 1–2 min per voltage; pulse duration 0.4 ms). The area under the curve (AUC) (g min^{-1}) and the amplitude of contractions (g) from the baseline were measured to estimate mechanical activity.

Drugs and solutions

The composition of the Krebs solution was as follows (in mM): glucose 10.10, NaCl 115.48, $NaHCO_3$ 21.90, KCl 4.61, NaH_2PO_4 1.14, $CaCl_2$ 2.50 and $MgSO_4$ 1.16 bubbled with a mixture of 5% CO_2-95% O_2 (pH 7.4). The following drugs were used: nifedipine, phentolamine, Nω-nitro-L-arginine (L-NNA), adenosine 5′-[β-thio]diphosphate trilithium salt (ADPβS), sodium nitroprusside (NaNP), atropine sulphate (Sigma Chemicals, St. Louis, MO, USA); (1R,2S,4S,5S)-4-[2-Iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt (MRS2500) and propranolol (Tocris, Bristol, UK). Stock solutions were prepared by dissolving drugs in distilled water except for nifedipine, which was dissolved in 96% ethanol, and L-NNA that required sonication to be dissolved in Krebs solution.

Data analysis and statistics

Nonlinear regression was used to fit experimental data to mathematical equations. Mechanical data were expressed as a percentage of the basal amplitude or basal AUC of contractions, while IJP amplitude is expressed in mV. Responses to drugs, voltage and frequency of EFS in the different regions of the colon were compared using a two-way ANOVA test followed by a Bonferroni's multiple comparisons test. Differences in tone, RMP and SD of tracings representative of spontaneous IJP (Gil *et al.* 2013) were compared using a one-way ANOVA followed by a Bonferroni's multiple comparisons test. Data were considered significant when $P < 0.05$. n values represent samples from different animals.

Results

Purinergic and nitrergic IJP characterization in the different colonic regions

The RMP did not differ between regions (one-way ANOVA, n.s.). Single pulses of EFS at 28 V and a total duration of 0.4 ms elicited a biphasic IJP (a IJPf followed by a IJPs) in all preparations. However, the amplitude of the IJPf and the IJPs seemed to differ between regions. While the proximal colon displayed a small amplitude IJPf followed by a large amplitude IJPs (Fig. 1a), the distal colon displayed a large amplitude IJPf followed by a very small amplitude IJPs (Fig. 1c). In the mid colon, the amplitude of both IJPf and IJPs was intermediate between that observed in the proximal and distal colon (Fig. 1b).

The gradient of inhibitory responses was confirmed after isolation of the purinergic component using L-NNA 1 mM (Fig. 1e) and the nitrergic component with MRS2500 at 0.3 μM (Fig. 1f). MRS2500 concentration was established after performing a concentration–response curve (from 0.3 nM and 0.3 μM in L-NNA 1 mM incubated tissue) in the mid colon (Fig. 1d). The amplitude of the maximum purinergic ($IJPf_{MAX}$) and nitrergic ($IJPs_{MAX}$) response elicited with a single pulse followed the rank of order for the purinergic response distal (−35.3 ± 2.2 mV) > mid (−30.6 ± 1 mV) > proximal colon (−11.7 ± 1.1 mV) and proximal (−11.4 ± 1.1 mV) > mid (−9.1 ± 0.4 mV) > distal colon (−1.8 ± 0.3 mV) for the nitrergic response. A two-way ANOVA followed by a Bonferroni *post hoc* test showed significant differences at the higher voltages between the proximal vs. mid and distal colon for purinergic neurotransmission ($P < 0.0001$) and between the distal vs. proximal and mid colon for nitrergic neurotransmission ($P < 0.0001$).

Effect of frequency on purinergic and nitrergic IJP in different colonic regions

To evaluate the effect of increasing the stimulation frequencies on purinergic responses, the tissue was

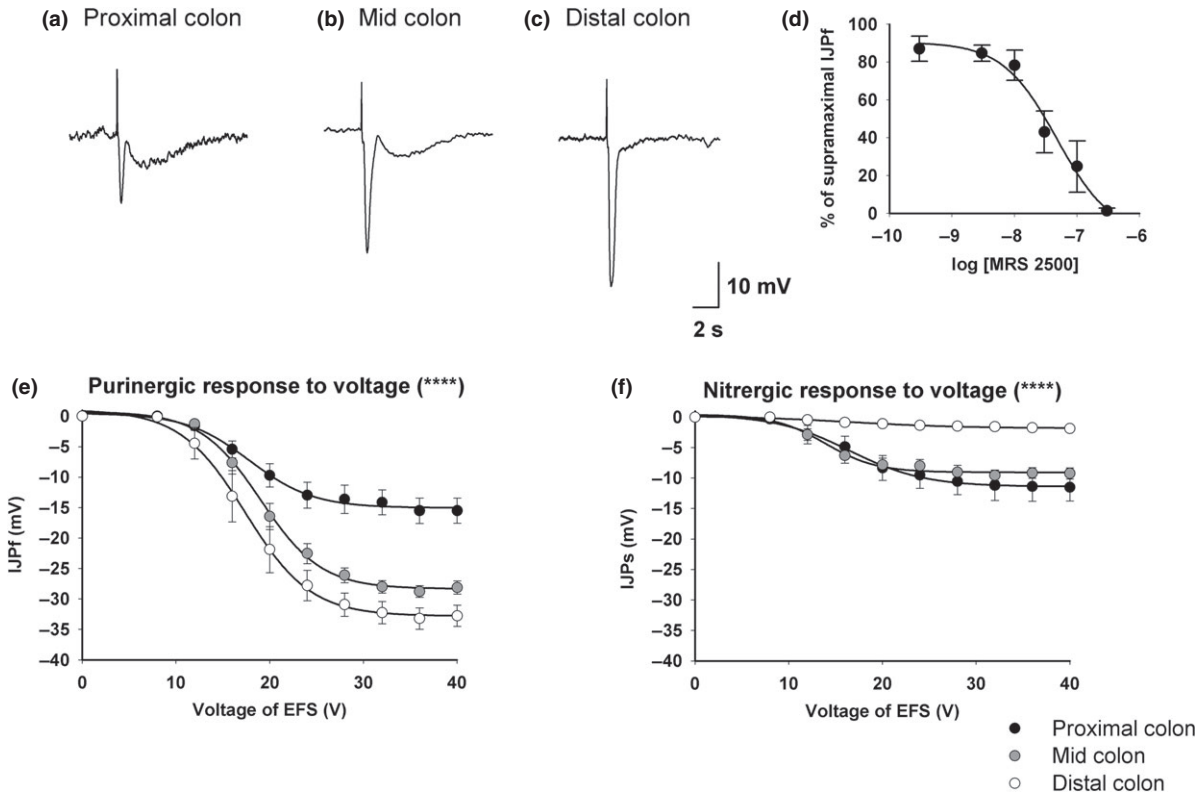

Figure 1 The $IJPf_{MAX}$ increases distally, while the $IJPs_{MAX}$ increases proximally. Recording of an IJP elicited with a single pulse (28 V, 0.4 ms) in the proximal (a), mid (b) and distal colon (c) with both the IJPf and IJPs. (d) Concentration–response curve for MRS2500 in the presence of L-NNA 1 mM in the mid colon (n = 5; R^2 = 0.85; $\log EC_{50}$ = −7.3 ± 0.2). Notice that the IJPf was totally abolished by MRS2500 at 0.3 μM. Effect of increasing the voltage of electrical field stimulation (EFS) from 0 to 40 V on the IJPf (e) and IJPs (f) amplitude in the proximal, mid and distal colon in the presence of L-NNA 1 mM and MRS2500 0.3 μM respectively. Data were fitted to sigmoid voltage–response curves (proximal colon: purinergic D.F. = 106; R^2 = 0.6; n = 11 nitrergic D.F. = 86; R^2 = 0.4; n = 9, mid colon: purinergic D.F. = 156; R^2 = 0.9; n = 18 nitrergic D.F. = 106; R^2 = 0.6; n = 11, distal colon: purinergic D.F. = 106; R^2 = 0.8; n = 11 nitrergic D.F. = 96; R^2 = 0.3; n = 11).

incubated with L-NNA 1 mM. The frequency increase progressively reduced the amplitude of the purinergic phasic hyperpolarizations, leaving a residual purinergic response at high frequencies ($IJPf_{\infty}$) (Fig. 2). This response fitted an exponential curve with a frequency-dependent decay (Fig. 2d) that is referred to as purinergic *rundown* (King 1994, Matsuyama *et al.* 2003, Gallego *et al.* 2008). The amplitude of the residual purinergic hyperpolarization followed the same rank of order than the maximum response reached with single pulses, being bigger in the distal colon (−10.3 ± 1.3 mV) (Fig. 2c), intermediate in the mid colon (−6.8 ± 1.2 mV) (Fig. 2) and smaller in the proximal colon (−5.1 ± 0.8 mV) (Fig. 2a).

To test the effect of frequency increase on nitrergic responses, the tissue was incubated with MRS2500 0.3 μM. The nitrergic response progressively increased with frequency-increasing EFS due to an additive effect of single IJPs reaching a maximum response at high frequencies ($IJPs_{\infty}$) and fitting an exponential curve with a frequency-dependent increase (Fig. 2d). Similar to that observed with the voltage-increasing trains, the mean amplitude of the sustained nitrergic hyperpolarization followed the rank of order: proximal (−28.9 ± 1.8 mV) (Fig. 2a) > mid (−21.9 ± 1.7 mV) (Fig. 2b) > distal colon (−9.3 ± 1.3 mV) (Fig. 2c).

At equal frequencies, the amplitude of electrophysiological responses obtained with isolated trains of EFS (20 s) was identical to the amplitude of inhibitory responses in continuous frequency-increasing trains of EFS. This means that, for example, the attenuation of or increase in the response obtained with an isolated train at 2 Hz was the same that the one recorded at 2 Hz in continuous frequency-increasing trains (not shown). Therefore, as observed in the rat and human colon (Mane *et al.* 2014a,b), responses are not affected by previous frequencies of stimulation.

A two-way ANOVA followed by a Bonferroni *post hoc* test again proved that significant differences exist between the proximal vs. mid and distal colon for the

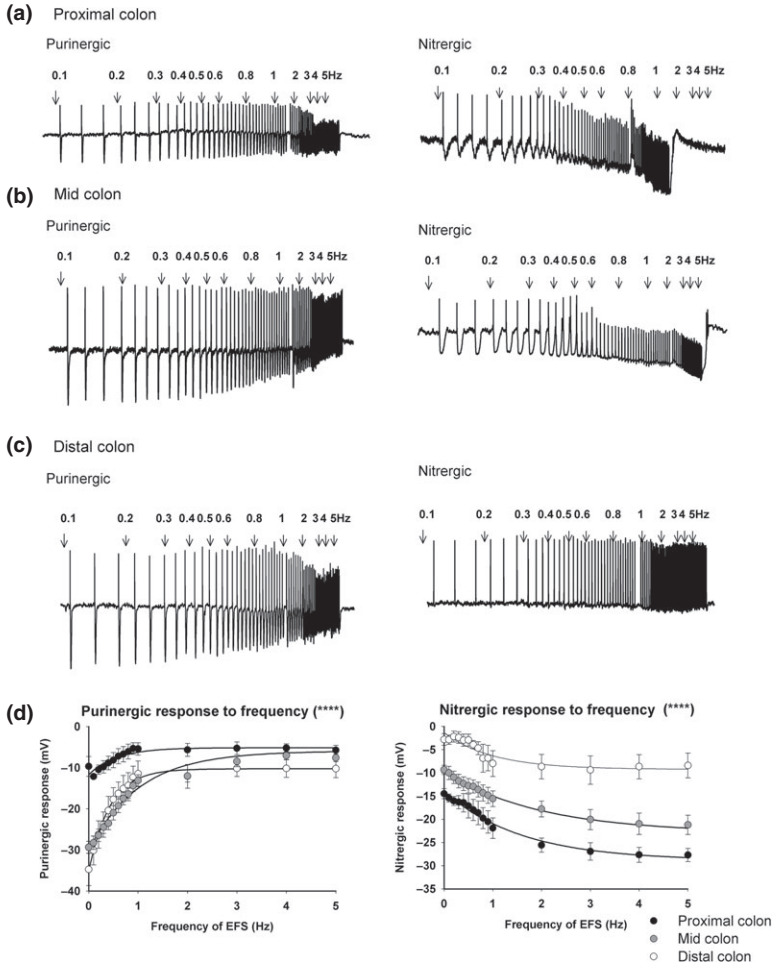

Figure 2 Frequency raise has an opposite effect on IJPf and IJPs in all regions. Microelectrode recording of a frequency-increasing train (from 0 to 5 Hz) in the proximal (a), mid (b) and distal colon (c) in the presence of L-NNA 1 mM (left) and MRS2500 0.3 μM (right). The total duration of the train is 2 min; the frequency is increased when the amplitude of the inhibitory response is stable. Increasing frequencies lead to purinergic *rundown* and a nitric oxide (NO)-mediated sustained hyperpolarization of smooth muscle cells (SMCs). (d) Graphical representation of the effect of increasing frequencies on IJPf (left) and IJPs (right) fitting exponential curves (proximal colon: purinergic D.F. = 57; R^2 = 0.3; n = 4 nitrergic D.F. = 57; R^2 = 0.6; n = 4, mid colon: purinergic D.F. = 132; R^2 = 0.7; n = 9 nitrergic D.F. = 102; R^2 = 0.4; n = 7, distal colon: purinergic D.F. = 57; R^2 = 0.7; n = 4 nitrergic D.F. = 57; R^2 = 0.3; n = 4).

purinergic component ($P < 0.0001$) and between the distal vs. proximal and mid for nitrergic neurotransmission ($P < 0.0001$).

Regional differences in the dynamics of the cotransmission process

The dynamics of purinergic–nitrergic cotransmission taking into account the isolated responses to voltage and frequency increase in EFS are represented, for each region, in Figure 3. The equations used for this graphical representation were obtained using the methodology previously described for the dynamics of inhibitory cotransmission in the rat and human colon (Mane *et al.* 2014a,b).

Purinergic response:

$$\mathrm{IJPf_V\,(mV)} = \mathrm{IJPf_{MAX}} - \frac{\mathrm{IJPf_{MAX}}}{1 + 10^{(\mathrm{Vf50-Vf})\times \mathrm{Hill\ slope\ (f)}}}$$

Nitrergic response:

$$\mathrm{IJPs_V\,(mV)} = \mathrm{IJPs_{MAX}} - \frac{\mathrm{IJPs_{MAX}}}{1 + 10^{(\mathrm{Vs50-V})\times \mathrm{Hill\ slope\ (s)}}}$$

Effect of purinergic and nitrergic neuromuscular cotransmission on spontaneous contractility

The effect of each inhibitory neurotransmission pathway on spontaneous motility was studied in each colonic region. Stimulation trains of 5 Hz frequency (see Fig. 3) at increasing voltages were used in order to

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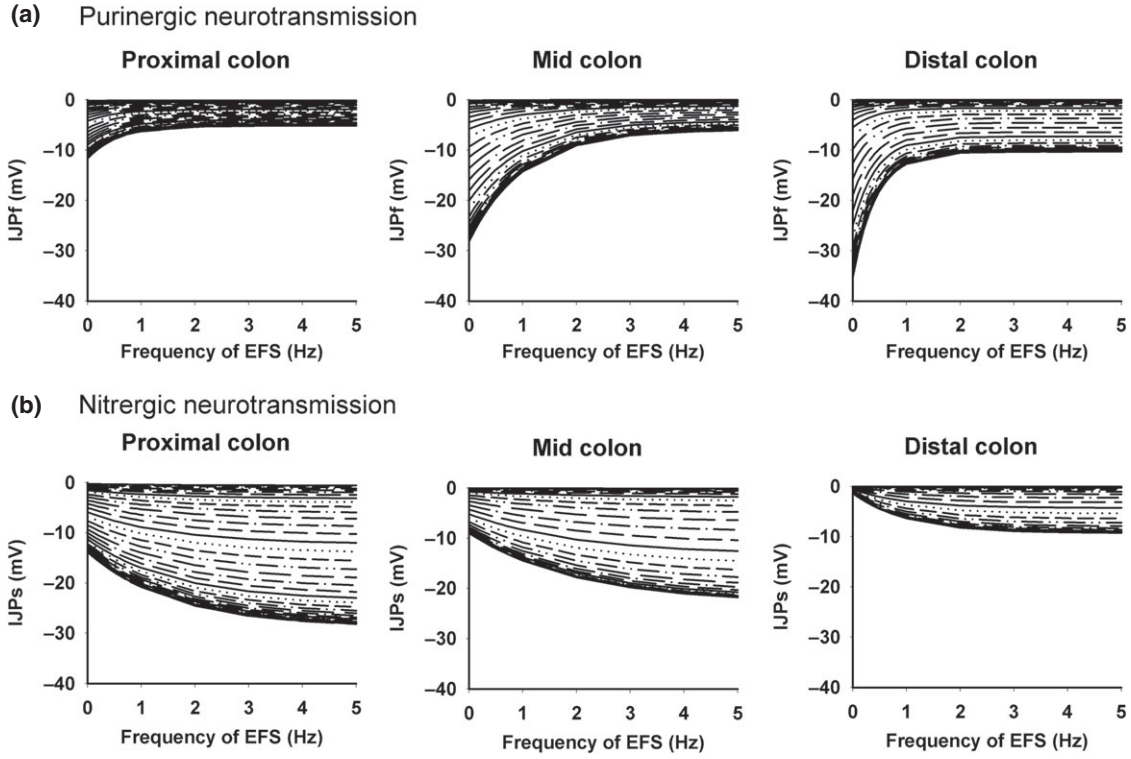


Figure 3 Modelling of inhibitory responses reveals an inverse gradient of purinergic and nitrergic neurotransmission through the mouse colon. (a) Representation of purinergic neuromuscular transmission in the proximal, mid and distal colon using experimental values. (b) Representation of nitrergic neuromuscular transmission in the proximal, mid and distal colon using experimental values. The different lines in each graphic represent responses at different voltages of electrical field stimulation (EFS).

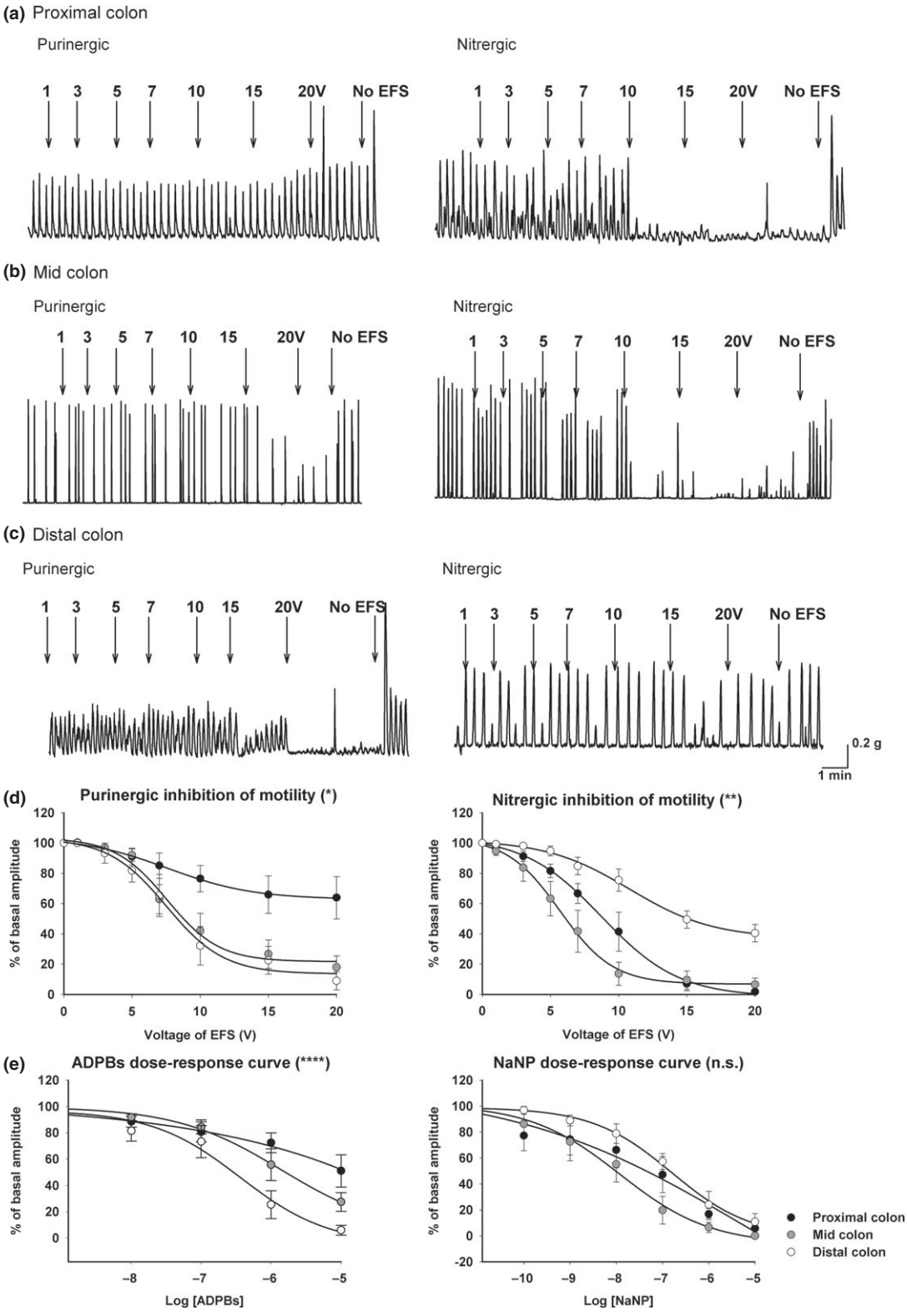
achieve a progressive inhibition of contractions. Experiments performed in the presence of L-NNA 1 mM showed that the inhibition of motility evoked by purinergic neurotransmission was greater in the distal colon (Fig. 4c), followed by the mid (Fig. 4b) and proximal colon (Fig. 4a). On the other hand, in the presence of MRS2500 0.3 μM, nitrergic inhibition of motility was higher in the proximal colon (Fig. 4a), followed by the mid (Fig. 4b) and distal colon (Fig. 4c).

A two-way ANOVA followed by a Bonferroni *post hoc* test proved that there were significant differences between the proximal vs. mid and distal colon for purinergic inhibition ($P = 0.0498$) and between all three regions in nitrergic inhibition of spontaneous motility ($P = 0.0028$).

We performed a concentration–response curve for the $P2Y_1$ receptor agonist ADPβs (from 10 nM to 10 μM, Fig. 4e, left) and the NO donor NaNP (from 0.01 nM to 10 μM, Fig. 4e, right) in proximal, mid and distal colon in order to test whether the differential purinergic and nitrergic responses observed along the colon were due to post-junctional sensitivity variance. The two-way ANOVA revealed no significant differences between regions concerning the NaNP concentration–response curves (Fig. 4e, right), suggesting that the variance in nitrergic neurotransmission among colon regions is not due to post-junctional events. In contrast, the ADPβs concentration–response curve differed significantly between the proximal and mid colon when compared to the distal colon (Fig. 4e, left, $P = 0.0003$), being the mechanical activity of the latter the most sensitive to the $P2Y_1$ agonist.

Inhibitory neural tone in different colonic regions

The purinergic and nitrergic tone in each colonic region was tested by measuring the AUC of spontaneous mechanical activity before and after the addition of either MRS2500 0.3 μM or L-NNA 1 mM respectively. The addition of MRS2500 0.3 μM to proximal (Fig. 5a, top) and mid colon preparations (Fig. 5b, top) had little effect, leading to increases in basal AUC of 22.3 ± 9.5 and 22.8 ± 8.8% respectively. In contrast, addition of MRS2500 0.3 μM to distal colon preparations (Fig. 5c, top) increased the basal AUC by 82.3 ± 14.7%, suggesting the presence of a significant purinergic tone in this region, which anticipates a constant release of endogenous purines in the distal colon. Regarding the nitrergic tone, addition of L-NNA 1 mM to the proximal (Fig. 5a, bot-



tom) and mid colon (Fig. 5b, bottom) increased the basal AUC by 493.6 ± 144.5 and 114.2 ± 26.6%, respectively, revealing an important spontaneous release of NO in this area. The nitrergic tone in the distal colon is practically inexistent, as addition of L-NNA 1 mM to preparations of this region (Fig. 5c, bottom) only increased motility an 11.2 ± 3.8%. A one-way ANOVA followed by a Bonferroni *post hoc* test

Figure 4 Inhibition of mechanical activity is achieved by different neurotransmitters in the different colonic regions. Shown are myographic recordings obtained with trains of increasing voltage (from 0 to 20 V) delivered at a frequency of 5 Hz in preparations of the proximal (a), mid (b) and distal colon (c) in the presence of L-NNA 1 mM (left) and MRS2500 0.3 μM (right). Voltage increases (1–2 min per voltage) lead to a progressive inhibition of the mechanical activity. (d) Graphical representation of the effect of increasing the electrical field stimulation (EFS) voltage on purinergic (left) and nitrergic (right) neurotransmission on mechanical activity (% of basal amplitude of contractions) fitting sigmoid curves (proximal colon: purinergic D.F. = 44; R^2 = 0.4; n = 6 nitrergic D.F. = 36; R^2 = 0.9; n = 5, mid colon: purinergic D.F. = 44; R^2 = 0.8; n = 6 nitrergic D.F. = 44; R^2 = 0.8; n = 6, distal colon: purinergic D.F. = 28; R^2 = 0.8; n = 4 nitrergic D.F. = 28; R^2 = 0.9; n = 4). (e) Sigmoidal concentration–response curves of ADPβs (left) and NaNP (right) in the different colonic regions (proximal colon: ADPβs D.F. = 26; R^2 = 0.5; n = 6; logEC_{50} = −5.9 ± 0.8 NaNP D.F. = 24; R^2 = 0.7; n = 4; logEC_{50} = −5.3 ± 0.5, mid colon: ADPβs D.F. = 26; R^2 = 0.8; n = 6; logEC_{50} = −5.9 ± 0.8 NaNP D.F. = 38; R^2 = 0.8; n = 6; logEC_{50} = −8.0 ± 0.4 distal colon: ADPβs D.F. = 16; R^2 = 0.8; n = 4; logEC_{50} = −6.5 ± 0.3 NaNP D.F. = 24; R^2 = 0.9; n = 4; logEC_{50} = −6.8 ± 0.4).

revealed significant differences between the proximal and mid colon vs. the distal colon for the purinergic tone (P = 0.0036) and between the mid and distal colon vs. the proximal colon for the nitrergic tone (P = 0.0018) (Fig. 5d).

L-NNA 1 mM and MRS2500 0.3 μM also caused different effects on the RMP in each colonic region (one-way ANOVA P = 0.0055). L-NNA 1 mM depolarized the membrane potential in the proximal (2.57 ± 0.24 mV), mid (2.85 ± 0.32 mV) and distal colon (1.31 ± 0.18 mV). MRS2500 0.3 μM did not modify the membrane potential of the proximal or mid colon but caused a significant depolarization in the distal colon (2.83 ± 0.70 mV). Comparison between spontaneous IJP was established by measuring the standard deviation (SD) of stable membrane potential recordings (Gil *et al.* 2013). Regional differences between proximal vs. distal colon in the absence and presence of MRS2500 were observed (one-way ANOVA P < 0.0001). Spontaneous IJP were prominent in the distal (SD = 1.15 ± 0.23; n = 7) compared with the proximal colon (SD = 0.52 ± 0.24; n = 7) (Bonferroni P < 0.001). Spontaneous IJP recorded in the distal colon were abolished by tissue incubation with MRS2500 0.3 μM (SD = 0.41 ± 0.05; Bonferroni P < 0.0001). In contrast, no differences in SD were observed before and after tissue incubation with MRS2500 in the proximal colon.

Cholinergic contractions in the different colonic regions

Excitatory responses were evaluated in NNNP conditions and in the presence of phentolamine and propranolol at 1 μM. The voltage increase from 0 to 30 V at a frequency of EFS of 30 Hz increased the amplitude of contractions fitting a sigmoid curve in all regions (Fig. 6d). All three regions displayed atropine-sensitive cholinergic contractions of a similar amplitude (two-way ANOVA n.s.).

Discussion

The different regions of the colon display different physiological functions. Simplifying, the proximal

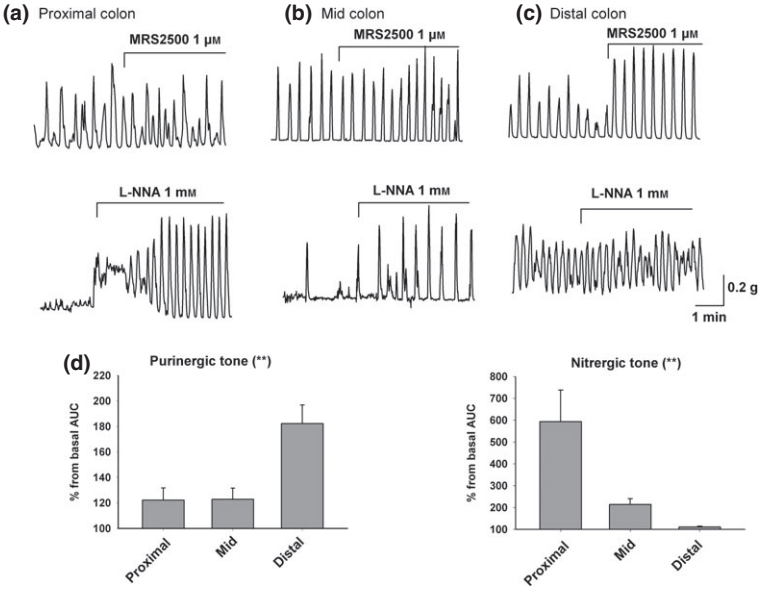


Figure 5 Purinergic and nitrergic inhibitory tone followed the same gradient than evoked neurotransmission. Myographic recordings before and after addition of MRS2500 0.3 μM (top) and L-NNA 1 mM (bottom) on proximal (a), mid (b) and distal colon preparations (c). (d) Histogram representing the effect of MRS2500 0.3 μM (left) and L-NNA 1 mM (right) addition on the area under the curve (AUC) of the proximal (MRS2500 n = 5; L-NNA n = 5), mid (MRS2500 n = 6; L-NNA n = 9) and distal colon (MRS2500 n = 4; L-NNA n = 7). The increase is expressed as a percentage of the basal AUC (AUC 2 min before drug addition vs. 2 min after drug addition).

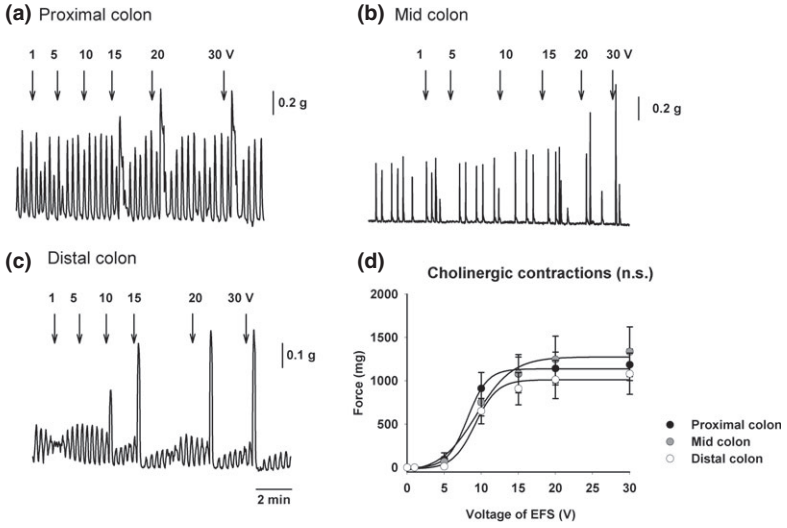


Figure 6 Cholinergic contractions were powerful along the colon. Myography recording of voltage-increasing trains of electrical field stimulation (EFS) (1–30 V, 30 Hz, 0.4 ms, 1 s) in NNNP conditions in the proximal (a), mid (b) and distal colon (c). (d) Histogram showing the sigmoidal voltage–response curves of each colonic region expressed in mg (proximal colon: D.F. = 108; $R^2 = 0.5$; $n = 16$, mid colon: D.F. = 122; $R^2 = 0.4$; $n = 18$, distal colon: D.F. = 108; $R^2 = 0.4$; $n = 16$).

colon acts mainly as a reservoir where most of the water and electrolytes are absorbed, while the distal colon functions mostly as a conduit that displays phasic and intense peristaltic contractions to propel faeces (Hertz & Newton 1913, Ritchie 1968, Snipes *et al.* 1982, Bassotti *et al.* 1987, 1989, Dapoigny *et al.* 1988, Christensen 1991, Ford *et al.* 1995, Dinning *et al.* 2012, 2014, Spencer *et al.* 2012). The aim of this work was to investigate whether a variation in the inhibitory cotransmission process and/or in cholinergic neurotransmission could be the basis of these regional differences in the colonic function.

Previous studies performed in our laboratory have shown that colonic relaxation is generated by the release of a purine and NO and that the frequency of stimulation probably mimicking neural firing of enteric inhibitory motor neurones is crucial for the predominance of one or another cotransmitter (Gallego *et al.* 2008, Mane *et al.* 2014a,b). Consequently, the relaxation pattern achieved at different neuronal firing frequencies might vary: single action potentials or short bursts lead to purinergic transient relaxations, while prolonged high firing frequencies possibly potentiate nitrergic neurotransmission, which is able to evoke a sustained inhibition of contractions. The present study confirms previous observation using short pulses showing that the IJPf amplitude is higher in the distal colon, whereas the IJPs is predominant in the proximal colon (Sibaev *et al.* 2003). Although interactions between nitrergic and purinergic pathways can exist (Van Crombruggen & Lefebvre 2004), proper isolation of each inhibitory neurotransmitter in combination with the adequate protocols of EFS was needed to properly characterize the relative contribution of each neurotransmitter in each colonic region. It is important to take into account that in the mice (not shown), rat (Mane *et al.* 2014b) and human colon (Mane *et al.* 2014a) summation of the electrophysiological responses in isolation is equivalent to the control hyperpolarization.

The behaviour of the purinergic IJPf and the nitrergic IJPs with voltage and frequency of EFS was proven to be practically identical to the one observed in the rat and human colon (Mane *et al.* 2014a,b). Despite that in all colonic regions both inhibitory components were observed, our experiments demonstrate that there are regional differences in the $IJPf_{MAX}$ and the $IJPs_{MAX}$ elicited by single pulses of EFS. The purinergic IJPf had higher amplitude in the distal colon in contrast to the nitrergic IJPs, which was larger in the proximal and mid colon and decreased distally.

Electrophysiological experiments performed with increasing frequencies of EFS showed that, in all colonic regions, the IJPf runs down to a plateau. However, the amplitude of the $IJPf_{\infty}$ reached at high frequencies varied between regions with the following rank of order: distal > mid > proximal colon. On the other hand, the nitrergic IJPs exponentially increased with frequency of stimulation leading to a sustained hyperpolarization of the SMC. Again, the amplitude of the IJPs achieved at high frequencies differed following the rank of order: proximal > mid > distal colon.

These regional differences in the purinergic and nitrergic inhibitory responses were confirmed using myographic contraction recordings. For instance, electric stimulation with the purinergic component isolated had little effect on spontaneous motility in the proximal colon, but it inhibited mechanical activity in the mid and distal colon. Contrarily, the nitrergic component was able to almost abolish spontaneous motility in the proximal and mid colon, but its inhibitory effect on the spontaneous contractions displayed by the distal colon was reduced. These results showing a proximal–distal decrease in nitrergic responses are in

accordance with the results obtained by Takahashi & Owyang (1998) in the rat colon, where nitrergic neurotransmission also seemed to be greater in the proximal compared with the distal colon. According to these results, a clear correlation between electrophysiological and mechanical responses exists along the colon. However, despite possible differences in IJP amplitude, if the value of membrane potential in post-junctional cells is below −40 mV, spontaneous contractions will be inhibited as they are extremely dependent on L-type calcium channels. Therefore, more negative values of the membrane potential should correlate with a major chance to have more cells in a relaxed state. Moreover, besides electromechanical coupling, pharmacomechanical coupling involving Gc-activated PKG acting on the contractile apparatus should also be considered for nitrergic neurotransmission.

A motility pattern that is important for determining the reservoir capacity and favouring the mixing and propulsion movements in the colon is the neural tone, that is action potential-driven TTX-sensitive release of neurotransmitters without the need of neural stimulation. *In vitro*, colonic preparations display an important inhibitory neural tone that until recently was considered to be predominantly due to the spontaneous release of NO (Gil *et al.* 2010). However, our data showed that in the distal colon, but not in the proximal and mid colon, there is a purinergic inhibitory tone as the addition of MRS2500, which blocks $P2Y_1$-mediated neuromuscular transmission, increased the AUC and spontaneous motility. MRS2500 also depolarized the RMP of distal colon preparations and abolished spontaneous IJP. The fact that purinergic neurotransmission suffers a rundown may seem to contradict the presence of a purinergic tone able to inhibit spontaneous motility. Previous results obtained in the human colon suggested that the rundown is due to a post-junctional desensitization as exogenous addition of a $P2Y_1$ agonist during the rundown did not further hyperpolarize the SMC (Mane *et al.* 2014a). However, pre-junctional inhibitory mechanisms, such as the pre-junctional inhibition of purine release, should also be considered (King 1994, Wang *et al.* 2015). In both cases and according to our results, it is plausible that purine release in the distal colon is able to keep the muscle hyperpolarized and maintain spontaneous contractions partially inhibited. On the other hand, the high relaxed state of the proximal and mid colon is entirely mediated by the spontaneous release of NO. In our electrophysiological studies, L-NNA significantly depolarized the three preparations, although the depolarization achieved in the distal colon was significantly lower.

The following question was whether the differences in purinergic and nitrergic inhibitory responses observed along the colon were caused by pre- or post-junctional events. The experiments here performed suggest that the larger purinergic response in the distal colon is at least in part due to post-junctional reasons, as the concentration–response curve for ADPβs in this area of the colon was shifted to the left. A logical hypothesis could be that $P2Y_1$ receptors are more abundant in this region of the colon, although a distally increasing purine release cannot be ruled out. Fibroblast-like cells (PDGFRα^+ cells) have been reported to be the intermediary step between enteric neurones and SMCs for purinergic neurotransmission. These cells express abundant $P2Y_1$ receptors, and all the intracellular mechanisms needed to transduce purinergic signals (Kurahashi *et al.* 2011, Baker *et al.* 2013). Moreover, calcium transients have been recorded in these cells after neuronal calcium increase (neurotransmitter release) and before SMCs rebound calcium rise, suggesting that the hyperpolarization takes places in the PDGFRα^+ cell and is then conducted to SMCs through gap junctions (Baker *et al.* 2015). Therefore, it would be interesting in the future to perform an experiment to assess whether there is an increase in PDGFRα^+ cells and/or $P2Y_1$ receptors throughout the length of the colon. Concerning nitrergic neurotransmission, mediated by the activation of guanylyl cyclase present in smooth muscle and interstitial cells of Cajal (ICC) (Lies *et al.* 2013, 2014), our data suggest that there are no differences in the post-junctional mechanisms responsible for NO-induced relaxation as the NaNP concentration–response curves were not statistically different between the different colonic regions. In the rat distal colon, both the population of NOS-reactive cells and NOS protein and mRNA levels were low compared with the proximal (Takahashi & Owyang 1998) and mid (Alberti *et al.* 2005) colon. This supports the statement that the higher nitrergic inhibition in the proximal and mid colon has its basis on pre-junctional events. However, this same study also suggested that the relaxant effect of exogenous NO was significantly reduced in the rat distal colon compared with the proximal colon and, therefore, it seems that there is also a post-junctional phenomenon responsible for the decreased nitrergic responses (Takahashi & Owyang 1998). Moreover, regional differences in the distribution of ICCs throughout the mouse colon have been reported. The density of intramuscular ICC varies considerably, being greatest in the most proximal regions and reducing in number towards the distal colon (Ward *et al.* 2002).

The murine colon is widely used for the evaluation of the purinergic and nitrergic pathways in genetically

modified animals with mutations affecting ICCs (Ward *et al.* 2002, Klein *et al.* 2013), Gc (Lies *et al.* 2014) or PKG. While performing these experiments, it is very important to (i) use always the same colonic segment, the proximal colon is more suitable to study the nitrergic pathway, while the distal colon allows a better characterization of purinergic neurotransmission and (ii) use a frequency of EFS that allows the maximal response of each neurotransmitter (Fig. 3).

It would not be unreasonable to speculate that the same phenomenon of gradation of the inhibitory cotransmission process is taking place in the human colon. The results of the inhibitory cotransmission dynamics in the human colon performed in our laboratory were obtained from samples of sigmoid colon (Gallego *et al.* 2006, 2008, 2011, Mane *et al.* 2014a). This area of the human colon is anatomically and functionally equivalent to the distal colon of rats and mice. In fact, single pulses of EFS delivered to human sigmoid colon elicit purely purinergic responses, whereas stimulation at frequencies higher than 0.6 Hz is needed to evoke nitrergic IJPs (Mane *et al.* 2014a). This is in accordance with the results obtained in the murine colon, as the distal colon has a predominant purinergic component and a low nitrergic presence. If this is true, then it is conceivable that nitrergic neurotransmission in the human colon is probably predominant in the right and transverse colon, which also act as a storage region for ileal effluent to allow absorption (Ford *et al.* 1995). Studies performed in the human colon show that the right and transverse colon have a higher density of circular muscle ICCs than the left colon (Hagger *et al.* 1998), similar to what has been reported in the mouse colon (Ward *et al.* 2002). Characterization of inhibitory neurotransmission in these sections of the human colon should be performed to confirm this hypothesis.

In conclusion, the present study shows that (i) in the proximal and mid colon nitrergic inhibitory neuromuscular transmission is predominant as, in this area, a tonic smooth muscle relaxation is needed to increase the reservoir capacity and accommodate content for long periods and (ii) in the distal colon, inhibitory neuromuscular transmission is mainly purinergic as phasic and intense contractions and relaxations are needed to propel dehydrated faeces.

Conflict of interest

The authors declare no competing financial interests.

This work has been funded by the following grant: BFU2009-11118 and 2011 CTP 00032. Noemí Mañé (AP2012-0897) and Míriam Martínez-Cutillas (AP2010-2224) are supported by the Ministerio de Ciencia e Innovación (Spain). Diana Gallego is supported by the Instituto de Salud Carlos III, Centro de Investigación Biomédica en red de enfermedades hepáticas y digestivas (CIBERehd).

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CHAPTER 4

Interplay between myogenic pacemakers and enteric neurons determine distinct motor patterns in the rat colon

Neurogastroenterol Motil, **26**, 1508-1512.

Neurogastroenterology & Motility

Neurogastroenterol Motil (2014) **26,** 1508–1512 doi: 10.1111/nmo.12393

HOT TOPIC

Interplay between myogenic pacemakers and enteric neurons determine distinct motor patterns in the rat colon

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Key Messages

- Recently, segmentation activity in the small intestine has been associated to the wax and wane of slow waves as a result of modulation of slow wave amplitude by a lower frequency pacemaker.
- Electrophysiological and mechanical data were obtained from colonic strips of 21 rats.
- In the rat colon, slow waves decrease their amplitude (wane) with cyclic depolarizations associated with low-frequency (LF) contractions, displaying the 'wax and wane' phenomenon.
- We propose that three main colonic motor patterns can occur depending on the level of excitation of both smooth muscle cells and interstitial cells of Cajal: propulsion (cyclic depolarizations), ripples (slow waves) or segmentation (wax and wane).

Abstract

Waxing and waning of slow waves amplitude has been recently associated with a segmentation motor pattern in the murine small intestine. The 'wax and wane' phenomenon in this area of the gastrointestinal tract seems to be the result of modulation of slow waves by a second pacemaker of a lower frequency displayed by the interstitial cells of Cajal near the deep muscular plexus (ICC-DMP). In the rat colon, smooth muscle cyclic depolarizations causing low-frequency (LF) contractions (0.9 ± 0.1 cpm) occur together with slow wave activity associated to high-frequency (HF) contractions (14 ± 0.3 cpm; ripples). In the present manuscript, we demonstrate the presence of 'wax and wane' in rat colonic slow waves. Depolarization from the 'wax' to the 'wane' was 7.6 ± 1.2 mV, i.e., smooth muscle cells went from a resting membrane potential (RMP) of −50.0 mV to a RMP of −42.4 mV. The amplitude of the slow wave decreased from 14.0 ± 2.2 mV to 3.4 ± 0.7 mV. The wax and wane phenomenon occurred at 0.9 ± 0.1 cpm, coinciding with the frequency of cyclic depolarizations. Therefore, we hypothesized that the 'wax and wane' of slow waves in the rat colon could be the result of their interaction with the LF pacemaker. We describe three different myogenic motor patterns that depend on the level of smooth muscle and ICC excitation: (i) LF propulsive contractions, (ii) regular slow waves causing ripples, and (iii) a wax and wane pattern that may lead to segmentation. Different intra- and

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Received: 2 April 2014
Accepted for publication: 13 June 2014

extra-luminal inputs probably determine the dominating motor pattern in each area through the enteric nervous system.

Keywords *colon, enteric nervous system, interstitial cells of Cajal, segmentation, slow waves, 'wax and wane'.*

Colonic motor activity has been classically divided into two modes: propulsive contractions or mass movements and non-propulsive contractions or segmental activity.[1,2] Segmentation has been recorded from the colonic lumen by indwelling sensors as pressure waves representing non-propulsive, non-peristaltic contractions of colonic smooth muscle.[3] This segmental activity mixes and homogenizes the content while increasing its exposure to the mucosal surface in order to facilitate the absorption of water and salts.[4–6]

In a recent paper published in Nature Communications, Huizinga *et al.*[7] provide evidence for a coordinated role of two networks of interstitial cells of Cajal (ICC) in the segmentation motor activity of the small intestine. Rhythmic contractions were recorded together with constant amplitude slow waves of the same frequency. Decanoic acid evoked waxing and waning of intestinal contractions associated with a wax and wane pattern in the amplitude of slow waves. In the small intestine, the network of ICC close to the myenteric plexus (ICC-MP) is the one responsible for slow wave activity. Interestingly, calcium imaging recordings of ICC located near the deep muscular plexus (DMP) showed a lower frequency pacemaker that strongly correlated with the waxing and waning of slow waves. Therefore, they conclude that in the small intestine the segmentation motor pattern is the result of the modulation of slow wave activity by the ICC-DMP pacemaker.

Waxing and waning of slow waves in intestinal musculature results in high (wax) and low (wane) open probability for Ca^{2+} channels, and has been previously recorded in other areas of the gastrointestinal tract and species such as the cat small intestine,[8,9] the porcine ileum,[10] the rat duodenum[11] or the human colon.[12] Diamant and Bortoff already in 1969[13] suggested that the wax and wane phenomenon could be possibly attributed to the interaction of two competing pacemakers, but they believed these pacemakers were both the slow wave generating pacemakers.

C-kit immunohistochemistry in the colon reveals the coexistence of two major populations of ICC: the ICC-MP and the submuscular ICC plexus (ICC-SMP). In contrast to the small intestine, in the colon, the ICC-SMP is the origin of the nifedipine-insensitive slow wave activity in different species[14–16] (Fig. 1C). Slow waves are associated with high-frequency (HF) contractions displayed in rat colonic strips (13–15 cpm; Fig. 1Db). Strips devoid of ICC-SMP display nifedipine-sensitive cyclic depolarizations (Fig. 1A) associated with high-amplitude low-frequency (LF) contractions (0.5–1.5 cpm; Fig. 1B) possibly originated in the ICC-MP.[16] *In vivo*, cyclic propagating bursts of action potentials[17] cause anally long distance propulsive contractions at an equivalent frequency to *in vitro* cyclic depolarizations and LF contractions.[4,18] In the colon, propulsive contractions are therefore associated with cyclic depolarizations while slow waves likely generate rhythmic bidirectional (anally or orally) propagating ripples.[19]

In a recent paper we have investigated the interaction of enteric inhibitory neurotransmission with pacemaker activity in the rat colon.[20] Similar to the finding of Sha *et al.*[21] in the mouse colon, we have found evidence consistent with the existence of a transwall gradient in the rat colon: smooth muscle cells (SMC) next to the ICC-SMP have a resting membrane potential (RMP) of -50.0 ± 0.9 mV while preparations without SMP have a RMP of -40.7 ± 0.7 mV. Whenever tissue displaying slow waves is hyperpolarized (electrotonically, or in our case, through inhibitory neurotransmission) slow waves increase their amplitude. In contrast, the amplitude of slow waves is decreased with depolarization.[22,23] HF contractions therefore persist under inhibitory neurotransmission while LF contractions, as the underlying depolarizations responsible for this type of contractions are highly L-type calcium channel-dependent, are abolished with hyperpolarization.[20]

Electrophysiological ($n = 13$) and mechanical recordings ($n = 8$) were obtained from rat mid colonic strips. Experimental procedures are detailed in Mane *et al.*[20] All procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona. In our recordings of slow waves in tissue with intact SMP, we noticed the presence of two different patterns. In some recordings, slow waves had a constant frequency and amplitude that was maintained throughout the experiment (Fig. 1C). However, in 8 out of 13 recordings, 80% of the impalements displayed the 'wax and wane' phenomenon of slow waves (Fig. 1E). Furthermore, in 5 out of 8 recordings of the organ bath technique (Fig. 1F and G) wax and wane of contractions was observed during the equilibration period (during the 30 min after a tension of 2 g was applied to the colonic strips).

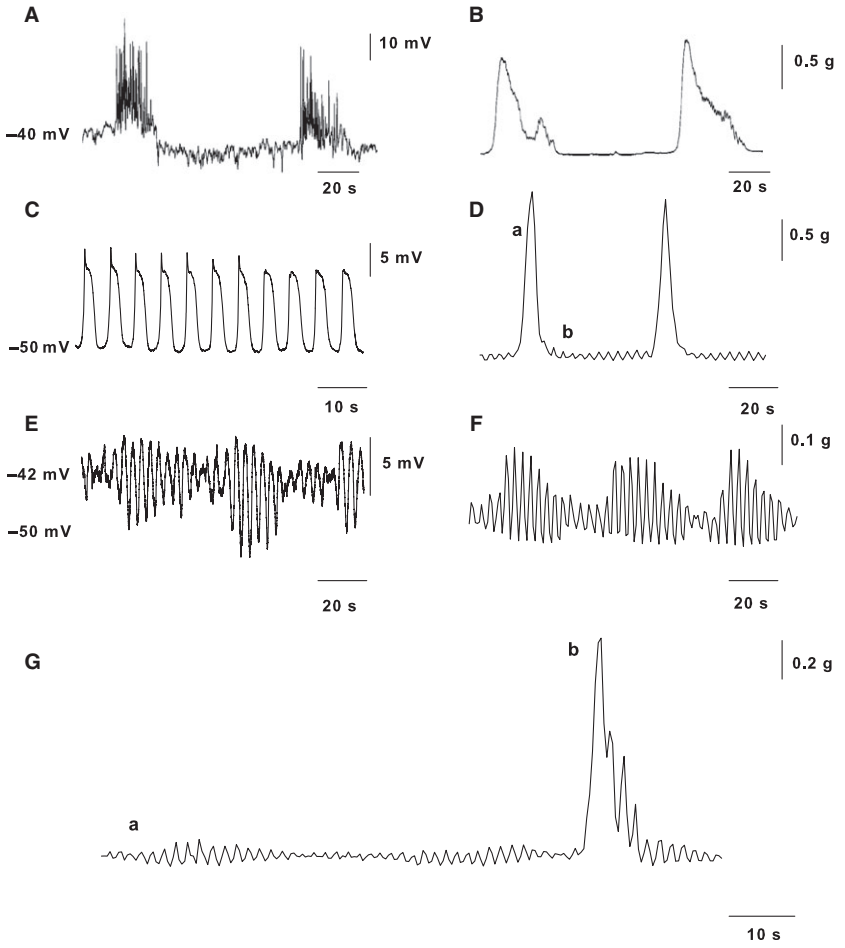

Figure 1 Electrophysiological and mechanical recording of cyclic depolarizations (A) and LF contractions (B) in ICC-SMP devoid tissue.[16] These recordings would be obtained when the tissue is depolarized (pattern 1). Notice the presence of spiking activity at the top of cyclic depolarizations generating LF contractions. (C) Electrophysiological recording of constant amplitude slow waves obtained in SMC with a low RMP (pattern 2). (D) Mechanical recording of LF (a) and HF contractions (b) after the equilibration period. (E) Electrophysiological and mechanical (F) recording of slow waves wax and wane (pattern 3). (G) Mechanical recording during the equilibration period of the wax and wane pattern and a LF contraction that appears with the same frequency as the previous mechanical wane phase.

After reading the article published by Huizinga *et al.*[7] where it is demonstrated that segmentation is due to the waxing and waning of slow waves as a result of their interaction with a lower frequency pacemaker associated with the ICC-DMP, we hypothesized that the slow wave waxing and waning of the rat colon could also be related to the LF pacemaker. Accordingly, in order to test this hypothesis, we measured the frequency of the 'wane' and compared it to the frequency of LF contractions (Fig. 1Da, E and F, values in Table 1). Frequencies were proven identical and, moreover, after the organ bath equilibration period we noticed that LF contractions replaced the wane phase of contractions (Fig. 1G). Therefore, we tried to find the electrophysiological explanation for this phenomenon reviewing our previous results.

As mentioned, when SMC displaying slow waves are depolarized, the amplitude of slow waves is known to decrease. In accordance, we measured the depolarization at the bottom of the slow wave from the 'wax' to the 'wane' and the amplitude of slow waves during each phase. Depolarization from the 'wax' to the 'wane' was 7.6 ± 1.2 mV, i.e., SMC went from a RMP of -50.0 mV to a RMP of -42.4 mV (both values measured at the bottom of the slow wave). Accordingly, the amplitude of the slow wave decreased from 14.0 ± 2.2 mV to 3.4 ± 0.7 mV (Fig. 1E and Table 1). Our opinion, based on these results, is that cyclic depolarizations responsible for the LF contractions are able to pull the membrane potential of SMC up, depolarizing the tissue and consequently reducing the amplitude of the slow waves generating the 'wane' pattern. When the cyclic depolarization finishes and the RMP returns slow waves can increase their amplitude or 'wax' (Fig. 1E).

Enteric nerves play an important role in this process as according to the level of neural excitation of both SMCs and ICCs, three patterns, similar to the ones recorded by Chen *et al.* in rat whole colon,[4] can occur:

Pattern 1: When the tissue is slightly depolarized by excitatory neurons or cessation of inhibitory

Table 1 Electrical and mechanical features of each predicted motor pattern*

Electrical features		Mechanical features		Motor pattern*
Cyclic depolarizations ($N = 6$)[16]		LF contractions ($N = 8$)		Propulsive contractions
1.4 ± 0.2 cpm	9.3 ± 1.6 mV	0.9 ± 0.1 cpm	4.3 ± 0.5 g	
RMP = −40 mV				
Wax and wane of slow waves ($N = 8$)		Wax and wane of HF contractions ($N = 5$)		Segmentation
0.9 ± 0.1 cpm		1.0 ± 0.1 cpm		
Wax	Wane	Wax	Wane	
RMP = −50 mV	RMP = −42 mV	0.21 ± 0.03 g	0.09 ± 0.02 g	
14.0 ± 2.2 mV	3.4 ± 0.7 mV			
Slow waves ($N = 13$)		HF contractions ($N = 8$)		Ripples
10.3 ± 0.5 cpm	14.0 ± 2.2 mV	14.7 ± 0.3 cpm	0.25 ± 0.04 g	
RMP = −50 mV				

Note that the frequency of the wane phenomenon and LF contractions associated to cyclic depolarization is coincident.RMP is measured at the bottom of slow waves or cyclic depolarizations. Values expressed in cpm correspond to the frequency of each pacemaker. Amplitude of slow waves and cyclic depolarizations is measured in mV and associated contractions in g.

neurotransmission, slow waves in SMCs tend to reduce their amplitude and cyclic depolarizations (Fig. 1A) and LF contractions (Fig. 1B) become dominant generating a propulsive motor pattern.

Pattern 2: When the tissue is slightly hyperpolarized by inhibitory neurons, the amplitude of the slow wave increases and a higher frequency propagating type of contractions become prevalent (Fig. 1C). This pattern possibly generates ripples enhancing absorption and appears between LF contractions (Fig. 1D) if they are present, as they tend to disappear with hyperpolarization due to their L-type calcium channel dependency.

Pattern 3: In an intermediary state, cyclic depolarizations modulate slow waves amplitude generating a wax and wane of contractions probably associated with a segmentation motor pattern (Fig. 1E and F). Probably, the beginning of the cyclic depolarization itself is able to trigger the wane of slow waves whereas the repolarization phase is associated with the wax. This interaction between pacemakers has been characterized as 'phase-amplitude coupling' in the small intestine, i.e., the amplitude of slow waves is modulated by the phase of cyclic depolarizations.[7]

Due to the transwall gradient and proximity to ICC-SMP, inner SMC have more possibilities to display pattern 2. In contrast, outer SMC being more depolarized will tend to display pattern 1. Cells in-between will preferentially display pattern 3 as they are equally coupled to both pacemakers. However, as SMCs and ICC are electrically coupled, the motility pattern displayed by the organ will depend on the level of excitability of SMC and specific innervation of ICCs, which will determine the relative strength of each pacemaker.

In conclusion, the ENS and pacemakers interplay in the establishment of a dominating motor pattern in the colon, being the LF pacemaker more susceptible to modification by neurotransmission than the HF pacemaker.[20] The omnipresent slow waves can be modulated by the LF pacemaker and the degree of modulation will depend on the ENS activity on ICCs and SMCs. Inhibitory and excitatory neurons will determine the level of excitability changing the motility pattern from propulsion (pattern 1) to ripples (pattern 2) or, the intermediate level, segmentation (pattern 3). Probably, several inputs such as the autonomic nervous system, the luminal content or even the type of content that reaches the colon determine spatiotemporal changes between motor patterns.

FUNDING

This work has been funded by the following grant: BFU2009-11118. Noemí Mañé (AP2012-0897), is supported by the Ministerio de Ciencia e Innovación (Spain). Marcel Jimenez is supported by the Instituto de Salud Carlos III, Centro de Investigación Biomédica en red de enfermedades hepáticas y digestivas (CIBERehd).

CONFLICTS OF INTEREST

The authors state no conflict of interest.

AUTHOR CONTRIBUTION

NM performed the research, analyzed the data and wrote the paper; MJ designed the research study and wrote the paper.

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CHAPTER 5

Enteric motor pattern generators involve both myogenic and neurogenic mechanisms in the human colon

Front Physiol, **6**, 205.

frontiers in Physiology

OPINION
published: 21 July 2015
doi: 10.3389/fphys.2015.00205

Enteric motor pattern generators involve both myogenic and neurogenic mechanisms in the human colon

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Keywords: human colonic motility, Interstitial Cells of Cajal, enteric neurotransmission, *in vitro* motility patterns, pacemaker

Edited by:
Jan D. Huizinga, McMaster University, Canada

Reviewed by:
Jan D. Huizinga, McMaster University, Canada
Phil Dinning, Flinders Medical Centre, Australia

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Specialty section:
This article was submitted to Autonomic Neuroscience, a section of the journal Frontiers in Physiology

Received: *24 April 2015*
Accepted: *06 July 2015*
Published: *21 July 2015*

Citation:
Mañé N, Martínez-Cutillas M, Gallego D and Jimenez M (2015) Enteric motor pattern generators involve both myogenic and neurogenic mechanisms in the human colon. Front. Physiol. 6:205. doi: 10.3389/fphys.2015.00205

Coordinated motor activity is required to develop the major functions of the colon, which are: 1-absorption of water, electrolytes, bile salts, short-chain fatty acids and other bacterial metabolites, 2-storage of colonic contents and 3-propulsion of fecal material (Christensen, 1991). Interstitial cells of Cajal (ICCs) generate spontaneous pacemaker currents which are conducted to smooth muscle cells (SMCs) causing rhythmic contractile patterns (Rumessen et al., 1993; Huizinga et al., 1995). Even though *in vitro* experiments disrupt enteric neural pathways crucial to develop a variety of *in vivo* colonic motor patterns and rule out any influence of extrinsic innervation, they are useful to better understand the mechanisms underlying colonic motility. Accordingly, the aim of this article is to summarize myogenic and neurogenic activities described in the human colon, hypothesize about how these mechanisms might be related and propose a new concept, *enteric motor pattern generators*, for this interplay.

Circularly-oriented strips from the human colon most commonly display low-amplitude contractions at an equivalent frequency to slow waves (**Figure 1A**). Strips that preserve the submucous plexus exhibit prominent 2–4 c.p.m slow waves that have their greatest amplitude near the ICC-submuscular plexus (ICC-SMP) (Rae et al., 1998). ICC-SMP are therefore responsible for colonic slow waves in the colon of animals (Langton et al., 1989; Pluja et al., 2001) and humans (Rumessen et al., 1993). Septal ICCs described in the human colon (Liu et al., 2012) might spread this pacemaker activity as previously suggested in the human small intestine (Lee et al., 2007a,b). *In vivo*, rhythmic phasic contractions (RPCs) at a frequency of 2–4 c.p.m are commonly recorded in the human large intestine (Taylor et al., 1975; Latimer et al., 1981; Narducci et al., 1987; Ford et al., 1995) strongly correlating to their frequency *in vitro*. The classical view states that the main role of RPCs is the turning over and mixing of luminal contents. However, recent studies using high resolution manometry have shown that RPCs can propagate anally or orally over short distances possibly causing propulsion (Dinning et al., 2014).

RPCs can appear together with high-amplitude contractions of a lower frequency (**Figure 1B**). When Rae et al. (1998) removed the submucosal plexus from human colonic strips leaving the myenteric plexus intact, strips displayed tetrodotoxin (TTX)-insensitive high-amplitude contractions at a frequency of 0.3–0.6 c.p.m. A similar contractile pattern has been reported in rat and mouse colonic strips when the ICC-SMP is removed (Pluja et al., 2001; Domenech et al., 2011). These results demonstrate the presence of a second myogenic pacemaker not dependent on the ICC-SMP or the enteric nervous system. The most probable origin of this pacemaker is the ICC network located near the myenteric plexus (ICC-MP) (Pluja et al., 2001; Carbone et al., 2013). In colonic large segments of different species, propulsive contractions at an identical frequency can be recorded. These propulsive contractions are inhibited after neural blockade but they can be restored

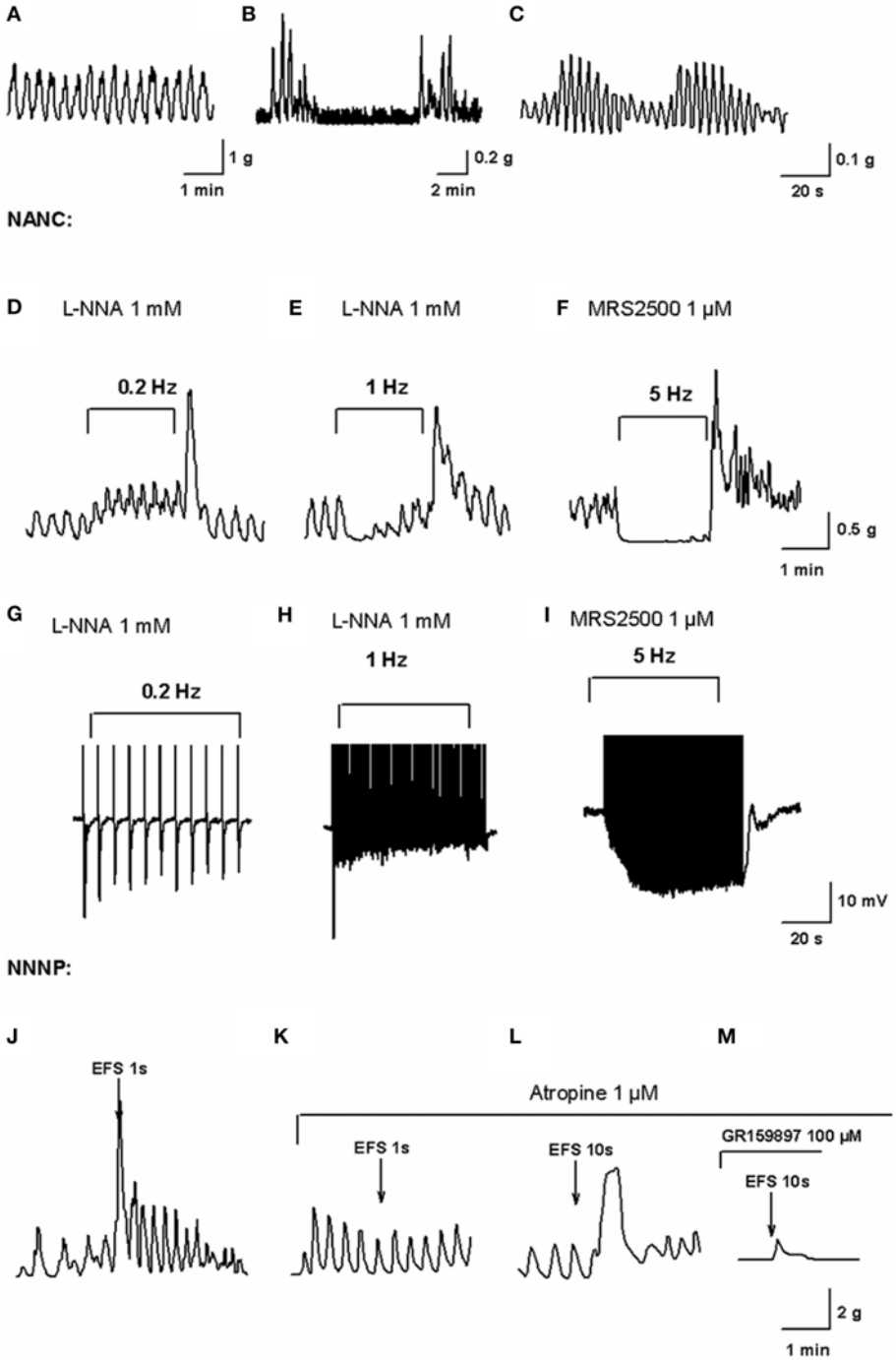

FIGURE 1 | ***In vitro*** **motility patterns and enteric neurotransmission in the human colon.** Mechanical recording of high frequency (HF) contractions of a constant amplitude probably associated to slow wave activity **(A)**, low-frequency (LF) contractions superimposed to HF contractions **(B)** and wax and wane of HF contractions amplitude **(C)** obtained from the experiments performed in our lab (Mane et al., 2014a). Mechanical recording of EFS under NANC conditions on L-NNA 1 mM incubated tissue at a frequency of 0.2 Hz **(D)** and 1 Hz **(E)** and MRS2500 1 µM incubated tissue at a frequency of 5 Hz **(F)** (Mane et al., 2014a). In L-NNA incubated tissue, purinergic neurotransmission is only able to cause phasic relaxations while nitrergic neurotransmission at 5 Hz completely inhibits spontaneous contractions. Electrophysiological recording of electrical field stimulation on L-NNA incubated tissue at a frequency of 0.2 Hz **(G)** and 1 Hz **(H)** and MRS2500 incubated tissue at a frequency of 5 Hz **(I)** (Mane et al., 2014a). Notice how purinergic fast IJP amplitude is reduced with high frequencies of EFS after the first pulse while the nitrergic response increases due to summation of slow IJP. Mechanical recording of EFS in NNNP conditions at 50 Hz, 50 V, 0.4 ms for 1 s **(J)** eliciting an atropine-sensitive **(K)** contraction. Mechanical recording of EFS in NNNP conditions at 50 Hz, 50 V, 0.4 ms for 10 s **(L)** eliciting an antiNK2-sensitive **(M)** contraction (Martinez-Cutillas et al., 2015).

with subsequent addition of carbachol demonstrating that myogenic propulsive contractions can occur in species such as the rat and rabbit colon (Huizinga et al., 2011; Costa et al., 2013). The ICC-MP receives excitatory neural inputs (Faussone-Pellegrini et al., 1990; Bayguinov et al., 2010) that may trigger the appearance of the low-frequency pacemaker *in vivo*. In *in vitro* conditions, the mechanisms responsible for the development of low-frequency contractions might be the mechanical stimuli elicited by the stretching of the strip (Huizinga et al., 2011).

Propagating spontaneous colonic motor complexes (CMCs) are the principal motor pattern occurring in *in vitro* recordings of whole isolated human colon (Spencer et al., 2012). They occur approximately every 4 min (0.25 c.p.m.), a frequency similar to the one of the low-frequency contractions mentioned above. Possibly due to the dimensions of the organ bath and the derived technical difficulties, no experimental evidences were provided about the neural origin of this activity, which has been confirmed in the mouse colon (Bywater et al., 1989).

High resolution manometry revealed mainly four contractile colonic motor patterns in humans (Dinning et al., 2014): cyclic motor patterns corresponding to RPCs (slow waves), short and long single propagating motor patterns and high-amplitude propagating contractions (HAPCs). Short and long single propagating contractions appear in intervals of more than 1 min, a frequency similar to the one displayed by low-frequency contractions recorded *in vitro*. HAPCs appear post-pandrially and only represent a small percentage of the total motor patterns recorded in healthy humans (6–10 HAPCs in 24 h, Bassotti and Gaburri, 1988; Rao et al., 2001; De Schryver et al., 2002). These high amplitude contractions develop a propulsive role (Cook et al., 2000), propelling intraluminal contents over large distances (Cannon, 1902). HAPCs can be activated by mechanical stimulation or chemical stimuli acting on underlying neural circuits which then initiate self-sustaining HAPCs. These activation mechanisms are similar to the ones that trigger low-frequency contractions in rat colonic segments. Once initiated, HAPCs cannot be blocked by lignocaine (Hardcastle and Mann, 1968) and therefore do not need a neural circuitry to develop after activation. We believe that the low-frequency pacemaker is the electrophysiological basis of short and long single propagating contractions and HAPCs. Luminal and extraluminal inputs may activate enteric neurons that in turn enhance the pacemaker possibly displayed by the ICC-MP. Consequently the force of contractions can increase from ≈23 mmHg of single propagating contractions to the ≈240 mmHg of HAPCs. Alternatively, inhibitory neurons can inhibit this myogenic pacemaker leaving RPCs as the predominant pattern.

As previously described in the rat colon (Mane and Jimenez, 2014), human colonic strips can display a "wax and wane" pattern of RPCs (**Figure 1C**) that can also be observed in the small intestine (see Figure 4 in Gallego et al., 2014). This motor pattern has been stated to be the basis of a segmentation-like pattern in the murine small intestine and the rat colon (Huizinga et al., 2014; Mane and Jimenez, 2014) and modulation of slow wave amplitude by a second pacemaker of a lower frequency has been proposed to be the underlying mechanism responsible. This hypothesis was raised due to the fact that both the ICC-deep muscular plexus of the murine small intestine and the ICC-MP of the rat colon display a pacemaker of an identical frequency to the wax and wane. Curiously enough, in the human colon the frequency of the wax and wane is similar to the frequency of low-frequency contractions. We therefore propose that in the human colon, the low-frequency pacemaker is the basis of the cyclic decrease in the amplitude of slow wave activity. Alternate contraction and relaxation of colonic segments separate the colon into chambers facilitating the contact between the intraluminal content and the colonic wall favoring absorption, mixing and slow propulsion of colonic contents.

The storage of colonic contents for long periods is accomplished by a sustained inhibition of contractile activity probably related to the presence of a neural inhibitory tone. Both in the human colon (Rae et al., 1998; Gallego et al., 2008) and laboratory animals (Alberti et al., 2005; Gallego et al., 2012), the addition of TTX usually increases the amplitude and frequency of spontaneous contractions *in vitro*. A similar effect is observed when colonic tissue is incubated with L-NNA or ODQ, a nitric oxide (NO) synthase and a guanylyl cyclase (Gc) inhibitor respectively. Therefore, a constant *in vitro* inhibition of spontaneous motility as a consequence of the "spontaneous" release of NO from enteric neurons has been reported. We believe that by stretching colonic strips in both electrophysiological and mechanical experiments, the release of NO is increased as the stretching may mimic the distention elicited by luminal content.

Excitatory and inhibitory neurons have, therefore, a role in the generation and/or development of the motor patterns explained above. While inhibitory neurotransmission is responsible for sustained relaxations of the colon, an active participation of excitatory neurotransmission has been proposed in the triggering of low-frequency contractions, development of CMC and HAPCs. Enteric innervation of the ICC networks can modulate the predominant motility pattern displayed by a certain colonic area in order to favor the development of the desired colonic function. Moreover, excitatory and inhibitory neurons can relax or contract the tissue independently of pacemaker activity.

In vitro, electrical field stimulation (EFS) of human colonic tissue elicits complex responses including contractions, relaxations and consequent off-contractions. These responses are the result of the simultaneous stimulation of all enteric neuronal pathways. As this might never happen *in vivo*, pharmacological conditions and parameters for selective stimulation of concrete neural pathways have to be established in order to emulate *in vivo* neurotransmission as accurately as possible.

Until now, in the human colon, enteric inhibitory neurotransmission has been shown to involve mainly purines and NO. It is well known that non-adrenergic, non-cholinergic (NANC) conditions are needed to study inhibitory neurotransmission. To further isolate purinergic and nitrergic responses, the NO synthesis blocker L-NNA or the $P2Y_1$ antagonist MRS2500 should be used. The frequency of EFS, which we believe mimics the firing frequency of neurons, has been stated to be crucial to enhance one or another component of inhibitory neurotransmission: while purinergic responses are dominant at low frequencies (<1 Hz) (**Figure 1G**) or short bursts (**Figure 1H**), high frequencies (>1 Hz) of EFS are needed

to release NO (**Figure 1I**) (Mane et al., 2014a,b). Purinergic responses are attenuated in a frequency-dependent manner and are therefore responsible for phasic relaxations required in propulsive activity (**Figures 1D,E**). NO, in contrast, can cause long-lasting inhibition of myogenic contractions (**Figure 1F**) and is therefore crucial for storage functions (see above).

On the other hand, the major neurotransmitters responsible for contractions released by enteric excitatory neurons are acetylcholine and tachykinins. Excitatory neurotransmission should be always characterized under non-nitrergic (L-NNA), non-purinergic (MRS2500) (NNNP) conditions and in presence of propranolol and phentolamine. In this case, the duration of the neuronal burst determines if the response is mainly cholinergic (short burst, 1 s) (**Figures 1J,K**) or if it also involves the release of tachykinins (long bursts, 10 s) (**Figures 1L,M**) (Martinez-Cutillas et al., 2015).

Direct innervation of SMCs by enteric motoneurons has been discussed over the last years. Interstitial cells have been proposed to mediate neurotransmission in the gut due to their proximity to nerve endings, their expression of receptors and signaling pathways for neurotransmitters and the existence of gap junctions with SMCs. Concerning excitatory neurotransmission, it has been shown that the ICC-MP receives excitatory input from motoneurons that release acetylcholine and tachykinins (Bayguinov et al., 2010) and that these cells are required for the mediation of cholinergic post-junctional responses. In nitrergic neurotransmission, guanylyl cyclase in both SMCs and ICC has been proven to be mandatory to induce a full nitrergic IJP (Lies et al., 2014). More recently, platelet derived growth factor receptor α positive (PDGFRα+) cells in colonic muscles (Kurahashi et al., 2012), which are also innervated by enteric inhibitory motoneurons, have been shown to mediate purinergic neurotransmission (Kurahashi et al., 2014). All these data have been provided using genetic modified mice that lack ICC or a certain post-junctional pathway, but the role of interstitial cells including PDGFRα+ cells in human tissue needs further evaluation.

Until now, the most studied intestinal motility pattern has been the peristaltic reflex. However, although a polarization of intrinsic neural pathways has been shown to exist in the human colon (Porter et al., 2002), very poor responses to acute distension have been reported using whole colonic preparations (Spencer et al., 2012). Therefore, it is unreasonable to believe that the peristaltic reflex is the unique basis of colonic motility. This would be like stating that spinal reflexes are the basis of movement. We believe that like spinal reflexes, peristaltic reflexes are only activated by particular stimuli. We propose a new concept, "enteric motor pattern generator" as an equivalent to the "central pattern generators" described in the spinal cord. In the gastrointestinal tract, different sublclasses of interstitial cells are the source of primary motility pacing contractions occurring at different frequencies. These patterns are constantly modulated by intrinsic and extrinsic innervation that provide the essential neural input to develop an efficient gastrointestinal motility.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DISCUSSION

ICCs and the ENS are the local regulators of gastrointestinal motility. ICCs establish the rhythm of contractions by cyclically depolarizing their RMP. The ENS through excitatory and inhibitory motor neurons brings closer or distances the smooth muscle from contraction respectively. Motor neurons can also, independently from the rhythm established by ICCs, produce contractions and relaxations in response to different luminal or extra-luminal stimuli that activate the enteric neural circuitry. Inhibitory motor neurons co-transmit NO and a purine, which, as shown in the present work, develop different functional roles and their distribution along the colon is in accordance with these differences.

The aim of the present thesis is to contribute to the knowledge of the regulation of gastrointestinal motility as the consequence of cooperation between the different cells types involved, focusing mainly on enteric inhibitory neurotransmission. The main questions we wanted to answer were the following: (1) Effect of the allosteric $P2Y_1$ antagonist BPTU on gastrointestinal purinergic responses (2) Dynamics of inhibitory co-transmission involving ATP or a related purine and NO (3) Functional role of each inhibitory co-transmitter (4) Distribution of purinergic and nitrergic neurotransmission along the colon (5) Effect of inhibitory co-transmission on gastrointestinal pacemakers and (6) Origin of the different colonic motility patterns observed in vivo.

NANC neurotransmission in the gastrointestinal tract

In the gastrointestinal tract, different experimental approaches have lead to the conclusion that enteric inhibitory neurotransmission is due to the release of ATP or a related purine and NO (Bult *et al.*, 1990;Burnstock *et al.*, 1970). Moreover, it is assumed that both neurotransmitters are released from the same inhibitory neuron and

that they are therefore co-transmitted. However, definitive data reinforcing this hypothesis has not yet been presented.

NO is a gasomediator and has, in consequence, the ability to diffuse through the plasma membrane of both the neuron and the post-junctional cell. The synthesis and release of NO requires an intracellular calcium increase as the activity of the enzyme of synthesis, nNOS, is calcium-dependent. Once in the post-junctional cell, NO hyperpolarizes and, therefore, distances the smooth muscle from the opening of calcium channels responsible for contraction, through activation of GC (Waldman & Murad, 1987;De Man *et al.*, 2007). The formation of cGMP activates PKG which phosphorylates different ionic channels. Nowadays, there is still an open discussion about the ion channels responsible for the nitrergic hyperpolarization, although the inhibition of chloride outward currents is gaining acceptance (Zhang *et al.*, 1998;He & Goyal, 2012;Gil *et al.*, 2012).

The role of ATP in the gastrointestinal tract was proposed by Burnstock in 1970 (Burnstock *et al.*, 1970). However, despite its early discovery, the post-junctional pathway activated by the purine has only been recently elucidated. The electrophysiological inhibitory response elicited by ATP was known to be apamin sensitive. Apamin is an $SK_{(Ca)}$ channel blocker that has been used widely to distinguish the two components of inhibitory electrophysiological responses: the first component, fast and of a high amplitude known as IJPf was apamin sensitive while the second component, more sustained and of a smaller amplitude termed IJPs was apamin insensitive (Zagorodnyuk & Maggi, 1994;Jimenez *et al.*, 1995;Serio *et al.*, 2003;Serio *et al.*, 1992;Vladimirova & Shuba, 1978;Xue *et al.*, 2000;Zizzo *et al.*, 2007;Crist *et al.*,

1992). Nevertheless, a recent study of our group showed that actually both inhibitory responses can be reduced by apamin, just with different relative sensitivity (Gil *et al.*, 2012).

The post-junctional pathway of ATP was therefore known to involve $SK_{(Ca)}$ channels (Vogalis & Goyal, 1997) but the receptor responsible for their activation was not discovered until 2006, when Gallego et al identified the $P2Y_1$ receptor as the one responsible for the IJPf with the use of $P2Y_1$ orthosteric antagonists such as MRS2179, MRS2279 and MRS2500 (Gallego *et al.*, 2006a;Gallego *et al.*, 2008;Grasa *et al.*, 2009). Afterwards, the involvement of the $P2Y_1$ receptor in enteric purinergic neurotransmission was confirmed by using $P2Y_1$ KO animals (Gallego *et al.*, 2012;Gil *et al.*, 2013;Hwang *et al.*, 2012).

The role of purinergic-nitrergic co-transmission in relaxation is not limited to the colon and, moreover, it has been observed in many species. This biphasic response is common in the human small intestine and colon (Gallego *et al.*, 2014;Gallego *et al.*, 2006b) and in species such as rodents and guinea pigs. In fact, we have published an article in which we demonstrate that this mechanism is also present in the horse pelvic flexure, an important site due to its involvement in many equine digestive diseases (Mas *et al.*, 2016) (Figure 1).

Purinergic and nitrergic functional neurotransmission is a highly conserved process of nerve-muscle relaxation in the gastrointestinal tract.

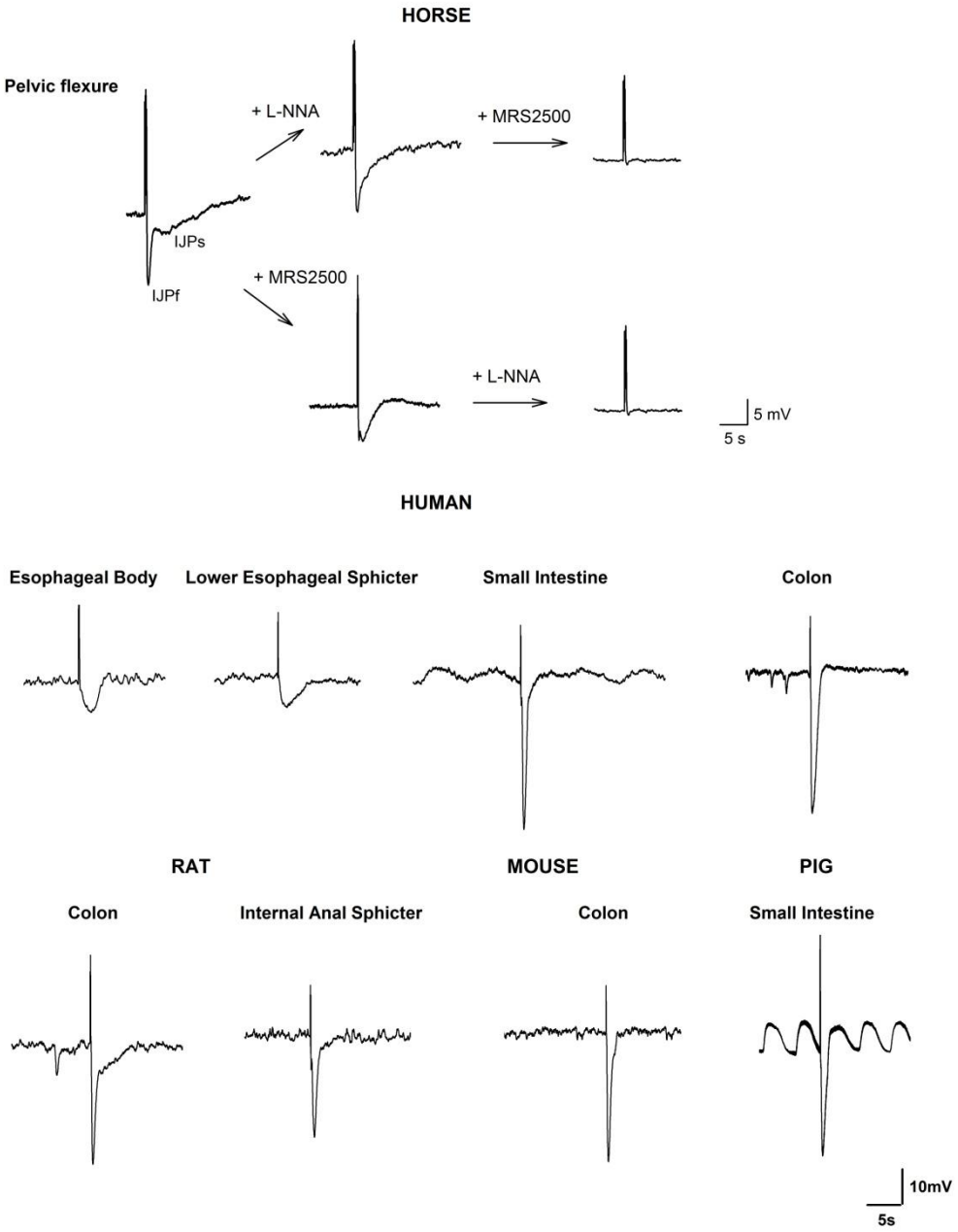

Figure 1. Purinergic and nitrergic neurotransmission in different species and regions. EFS in the equine pelvic flexure elicits a biphasic IJP composed by an MRS2500 sensitive IJPf followed by a IJPs sensitive to L-NNA (Mas *et al.*, 2016). Response to single pulses of EFS in different human gastrointestinal regions, rodents and pig. Modified from Jiménez et al., 2014 (Jimenez *et al.*, 2014).

BPTU effect on purinergic gastrointestinal responses (Annex)

$P2Y_1$ antagonists of a nucleotidic nature such as MRS2500 have been crucial for the identification of the receptor responsible for purinergic relaxation in the gastrointestinal tract. Recently, a non-nucleotide antagonist of the $P2Y_1$ receptor, BPTU, has been developed (Chao *et al.*, 2013). In the present work we demonstrated that BPTU blocked both electrophysiological and mechanical purinergic responses evoked by EFS, nicotine and $P2Y_1$ agonists (Image 1). Therefore, BPTU is able to reach the neuromuscular junction in the gastrointestinal tract and bind to the $P2Y_1$ receptor.

In a paper published in Nature Communications, it was described that the binding site of BPTU to the $P2Y_1$ receptor differs from previous nucleotidic antagonists in that it is located outside the orthosteric site (Zhang *et al.*, 2015), i.e., it is an allosteric drug. Safety advantages of BPTU versus MRS2500 have been demonstrated when used as an antithrombotic agent (Chao *et al.*, 2013). In fact, many allosteric drugs display safety advantages over orthosteric drugs (Tan *et al.*, 2006). One of the main reasons for this is that allosteric sites show greater divergence in the amino acid sequence between subtypes compared with the orthosteric domain, where the sequence is usually highly conserved. This allows increased subtype selectivity preventing the dose-limiting side effects of orthosteric agents.

Allosteric agents, in particular allosteric potentiators, depend on the endogenous orthosteric agonist for signalling. This characteristic confers another safety advantage over orthosteric agonists known as “effect ceiling” (Christopoulos & Kenakin, 2002), i.e., receptor activation beyond a certain point will not occur irrespective of drug

concentration, diminishing target-based toxicity. In addition, the dependence upon the orthosteric agonist for signalling makes allosteric potentiators ideal to preserve the physiological time and intensity of signals. Orthosteric exogenous agonists, in contrast, result in chronic activation which may end up in increased side effects or no effect due to a desensitization and/or internalization of the receptor, typical of G-protein coupled receptors (Bridges & Lindsley, 2008;Valant *et al.*, 2012;Nussinov & Tsai, 2014;Grover, 2013).

In conclusion, BPTU blocks $P2Y_1$ mediated responses in the gastrointestinal tract. This should be taken into account when considering its use as an antithrombotic agent. Allosteric modulation of G-protein coupled receptors is a promising approach for the treatment of diseases involving this type of receptors.

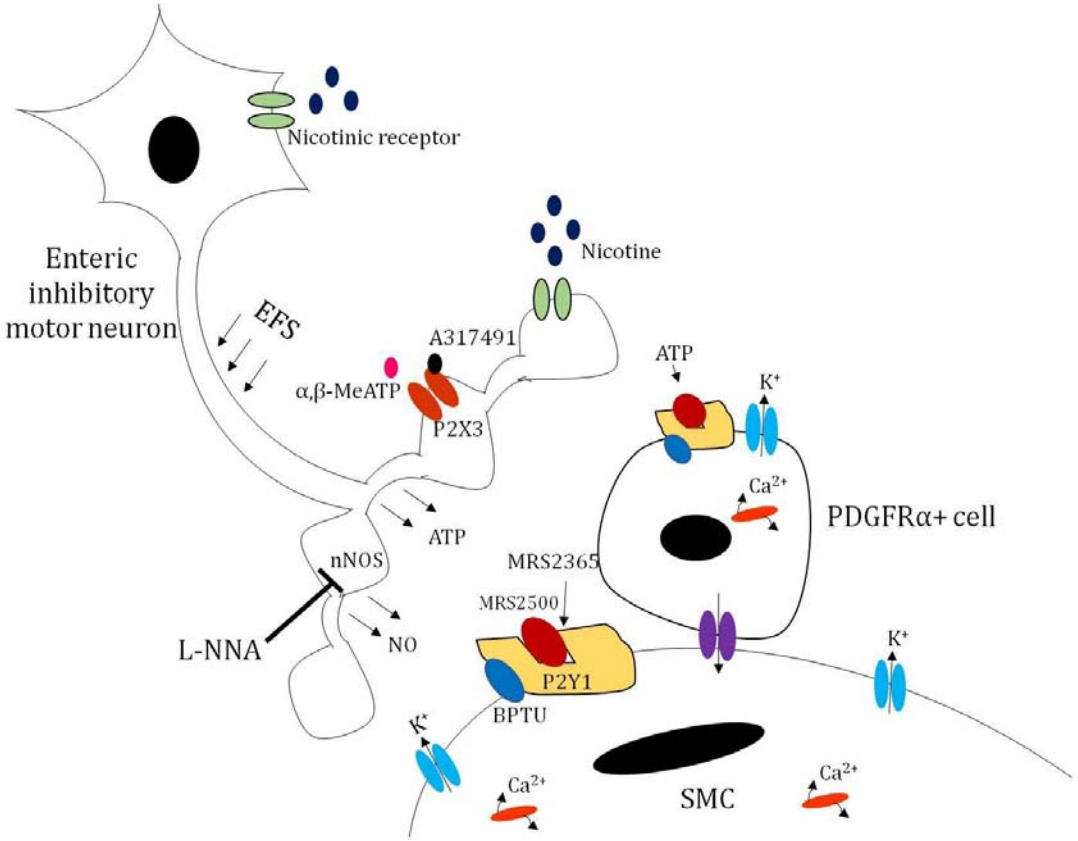

Image 1. BPTU is able to block colonic purinergic responses evoked by EFS, MRS2365 and nicotine. EFS and nicotine through nicotinic receptors evoke the release of ATP. MRS2365 activates the purine receptor directly. The response is blocked by MRS2500 and BPTU which bind orthosterically and allosterically to the $P2Y_1$ receptor. The $P2Y_1$ receptor is located in PDGFRα+ cells which then transmit the hyperpolarization to smooth muscle (SMC) through gap junctions. SMC also expresses $P2Y_1$ receptors. αβ-MeATP, a P2X agonist that has the same effects as $P2Y_1$ activation, may exert its action by stimulating the release of purines through the activation of an unidentified P2X receptor.

Now, what relevance would the blockage of the purinergic pathway have in gastrointestinal motor function if purinergic antagonists were used as a treatment? Would the nitrergic pathway compensate or do they have complementary (not redundant) functions? Are the released in the same circumstances? The next sections will focus on answering these issues.

Dynamics of inhibitory co-transmission in the colon

The identity of the two inhibitory neurotransmitters and their post-junctional pathway has been already described. The following question, was: *Are both neurotransmitters released in the same circumstances?*

Our approach was focussed on describing the putative different releasing stimuli that may account for the action of one or another neurotransmitter. By using different

parameters of EFS we wanted to mimic a varied range of experimental conditions and analyze the nature of the electrophysiological response.

We first used single pulses mimicking an action potential or a short train of action potentials in inhibitory neurons. As described previously, the rat and murine colon responded to single pulses of EFS with biphasic IJP consisting of a first IJPf followed by a IJPs. The human sigmoid colon, in contrast, displayed only a IJPf. The IJPf were blocked by the potent $P2Y_1$ antagonist MRS2500 while the IJPs were abolished with the nNOS inhibitor L-NNA. From this point, responses were studied separately, isolating purinergic responses with L-NNA and nitrergic responses with MRS2500. Single pulses were applied at increasing voltages of EFS leading to a progressive increase in post-junctional inhibitory responses. The human colon displayed no IJPs with single pulses. Both purinergic and nitrergic responses increase in a voltage-dependent manner, fitting a sigmoid curve, probably due to more recruited motor neurons or more neurotransmitter being released due to a superior calcium entry (Figure 2; Table 1 and 2). The maximal purinergic response obtained, the $IJPf_{MAX}$ was of greater amplitude than the $IJPs_{MAX}$ (Figure 2; Table 1 and 2).

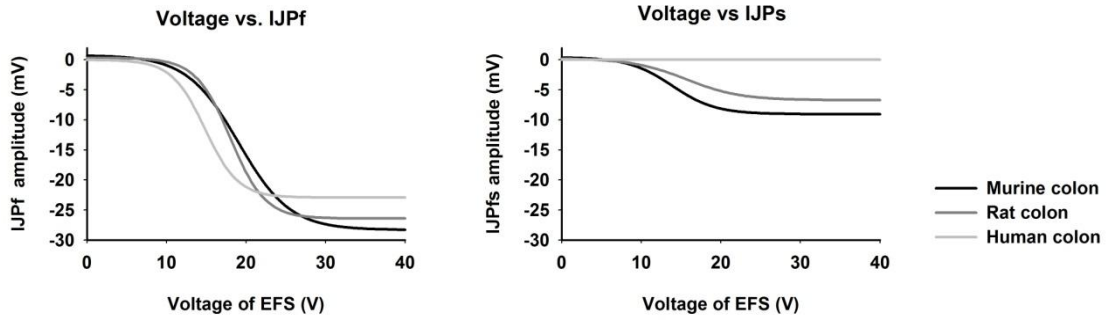

Figure 2. Effect of increasing voltages in the rat and murine mid colon and in the human sigmoid colon. Curves are based on the experimental data of the results section displayed in table 1 and 2.

The supramaximal voltage responsible for the IJP_{MAX} was then used to perform a protocol that consisted on increasing the frequency of EFS from 0.1 Hz to 10 Hz trying to mimic the endogenous firing frequency of inhibitory neurons. These frequencies were established with the little information available on the physiological firing frequencies of enteric neurons, which has been described to be between 0 and 15 Hz (Buhner *et al.*, 2009;Michel *et al.*, 2011). After isolating each inhibitory response, it was concluded that, in the three species, the behaviour of each neurotransmitter with frequency increase was opposite (Figure 3 and 4, Image 2).

Purinergic neurotransmission vs. Frequency of neuronal firing

Purinergic responses decreased their amplitude in a frequency-dependent manner until reaching a plateau that we have termed $IJPf_{\infty}$ as higher frequencies would not increase or decrease this response (Figure 3).

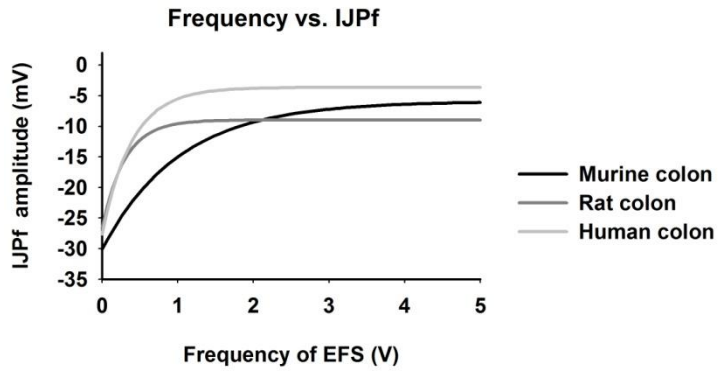

Figure 3. Effect of increasing frequencies on purinergic responses in the rat and murine mid colon and in the human sigmoid colon. Curves are based on the experimental data of the results section displayed in table 1.

The characteristics of the IJPf exponential fall for each species are illustrated in Table 1.

		Purinergic response		
		Rat	**Murine**	**Human**
Response to increasing voltages of EFS	***IJPf $_{MAX}$***	-26.4 ± 0.6 mV	-28.3 ± 0.8 mV	-27.6 ± 1.6 mV
	Vf50	18.0 ± 0.4 V	19.1 ± 0.5 V	15.8 ± 0.8 V
	Hill slope(f)	-0.20 ± 0.03	-0.13 ± 0.02	-0.17 ± 0.04
Response to increasing frequencies of EFS	***k (f)***	3.4 ± 0.6 s	1.2 ± 0.1 s	2.6 ± 0.3 s
	IJPf$_{\infty}$	-8.9 ± 0.6 mV	-7.6 ± 1.0 mV	-3.7 ± 0.6 mV

Table 1. Experimental values of purinergic neurotransmission in the rat and murine mid colon and in the human sigmoid colon.

The reason for the decrease of purinergic responses with frequency was further investigated. Two main reasons could account for the reduction of the purinergic response: (1) Pre-junctional reasons, such as a depletion of neurotransmitter or an auto or hetero-inhibition of the release or (2) Post-junctional reasons, such as desensitization of the post-junctional pathway. A very simple but clarifying experiment that consisted of adding a $P2Y_1$ agonist during a purinergic rundown was performed. If we solved the pre-junctional problem, i.e., neurotransmission depletion, by adding the "neurotransmitter" exogenously, no effect will indicate that this is not the reason for the rundown. No further hyperpolarisation was observed with the addition of the agonist which suggested that the post-junctional pathway is not able to respond to the neurotransmitter. In fact, experiments performed by other groups have shown that more purinergic neurotransmitter is released with higher frequencies of EFS ruling out the hypothesis of pre-junctional reasons (Durnin *et al.*, 2012;Hwang *et al.*, 2011;Mutafova-

Yambolieva *et al.*, 2007). $P2Y_1$ receptors are known to desensitize easily (Rodriguez-Rodriguez *et al.*, 2009;Hardy *et al.*, 2005). However, we tested the pathways of desensitization described and observed no modification or a very slow effect on the IJPf amplitude that could not explain the rapid decrease in the purinergic response that we observed. Therefore, the exact cause of the purinergic rundown is still unknown. One hypothesis is that the calcium increase in the post-junctional cell necessary for the activation of $SK_{(Ca)}$ channels is a limiting factor for a continuous purinergic signalling.

Nitrergic neurotransmission vs. Frequency of neuronal firing

Nitrergic responses, in contrast, increased their amplitude with frequency until reaching a maximum response ($IJPs_{\infty}$) (Figure 4). In the case of the rat and murine colon, the IJPs observed with single pulses added to the prior one when increasing the frequency generating a cumulative frequency-dependent exponential increase in the response (temporal summation). In the human colon, as no IJPs could be recorded with single pulses, only when high frequencies were reached (F0) a nitrergic hyperpolarization with an exponential increase with frequency could be recorded.

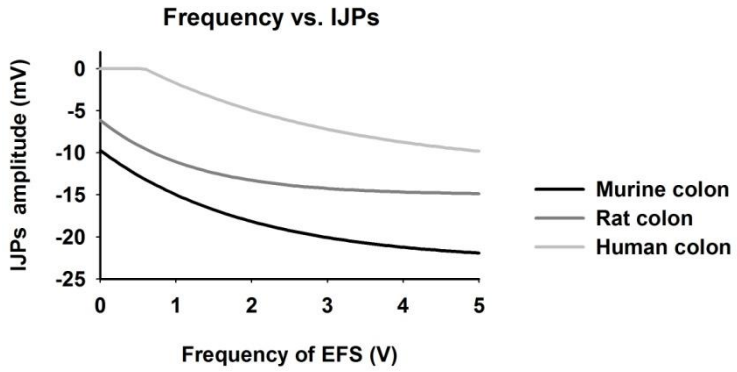

Figure 4. Effect of increasing frequencies on the nitrergic response in the rat and murine mid colon and in the human sigmoid colon. Curves are based on the experimental data of the results section displayed in table 2.

The characteristics of the IJPs exponential rise for each species are illustrated in Table 2.

		Nitrergic response		
		Rat	**Murine**	**Human**
Response to increasing voltages of EFS	***IJPs*** $_{MAX}$	-6.7 ± 0.3 mV	-9.1 ± 0.4 mV	
	Vs50	15.7 ± 1.0 V	13.9 ± 1.1 V	
	Hill slope (s)	-0.13 ± 0.03	-0.16 ± 0.05	
Response to increasing frequencies of EFS	***k (s)***	0.8 ± 0.2 s	0.6 ± 0.2 s	0.3 ± 0.1 s
	IJPs $_{\infty}$	-15.0 ± 0.5 mV	-21.9 ± 1.7 mV	-13.3 ± 0.4 mV

Table 2. Experimental values of nitrergic neurotransmission in the rat and murine mid colon and in the human sigmoid colon.

The combination of MRS2500 and L-NNA totally abolished inhibitory responses in the three species tested showing that a purine acting through $P2Y_1$ receptors and NO are the main inhibitory neurotransmitters in the colon in this range of frequencies. We do not have functional evidences suggesting the presence of other gaseous (H_2S, CO) or non gaseous (VIP) inhibitory neurotransmitters at least in our experimental conditions of nerve stimulation, which potentially mimic physiological parameters of neuronal firing.

In conclusion, the nature of the inhibitory response will be determined by the frequency of neuronal firing that probably varies depending on luminal and extra-luminal stimuli. Low firing frequencies or short stimuli will release mainly ATP or a related purine as long stimuli desensitize the purinergic post-junctional pathway. High firing frequencies recruit more calcium necessary for the synthesis and posterior release of NO.

In order to test the possible impairment of inhibitory neuromuscular transmission in the gastrointestinal tract of human samples or animal models it is therefore important to use an adequate protocol for each neurotransmitter. While purinergic neurotransmission should be tested using short stimuli, long duration pulses at high frequencies will better test the nitrergic component.

Functional role of each inhibitory co-transmitter

After stating that each inhibitory co-transmitter was acting at different frequencies of neuronal firing we wanted to answer the following question: *Why does the ENS need two inhibitory neurotransmitters?* Or, in other words, *do they have redundant, or complementary effects?*

In order to establish a functional role for each inhibitory co-transmitter, we performed mechanical experiments to observe their inhibitory effect on spontaneous motility. As in electrophysiological experiments, we isolated each inhibitory pathway using MRS2500 to study nitrergic inhibition and L-NNA to isolate purinergic neurotransmission.

We used our previous experimental data to determine the appropriate frequencies of stimulation. In the rat and mouse colon, we used frequencies of 5 Hz as at this frequency, the amplitude of each inhibitory neurotransmitter was sufficient to inhibit spontaneous motility. In the human colon, in contrast, due to its more pronounced rundown of purinergic responses and therefore, to the low amplitude of the $IJPf_{\infty}$, we started using lower frequencies in order to see the effect of pure purinergic responses.

Role of purinergic neurotransmission

Low frequencies of EFS in L-NNA incubated preparations showed how, after each pulse, purines were able to transiently inhibit spontaneous contractions. Afterwards, however, a rebound contraction totally dependent on the previous hyperpolarisation was observed. At high frequencies, in the three species, purines were only able to inhibit contractions at the beginning of the stimuli. Spontaneous motility started to recover when the maximal rundown of purinergic neurotransmission was reached.

Role of nitrergic neurotransmission

In MRS2500 incubated human colonic samples, low frequencies did not cause any effect on spontaneous contractility. This result is in accordance with no nitrergic electrophysiological responses in the human colon at low frequencies. At high

frequencies, NO was able to inhibit spontaneous contractions throughout the stimuli in all three species.

In conclusion, purinergic neurotransmission is only able to transiently inhibit spontaneous contractility and is therefore responsible for phasic relaxations. Meanwhile, NO is able to inhibit contractions in a sustained manner, being responsible for tonic relaxations.

Distribution of purinergic and nitrergic neurotransmission along the colon

After attributing a different physiological role for each neurotransmitter, phasic relaxations for purinergic neurotransmission and sustained relaxations for nitrergic neurotransmission, we elaborated the following hypothesis: *Nitrergic neurotransmission is probably dominant in proximal colonic regions while purinergic neurotransmission predominates in the distal colon*. This hypothesis was based on the fact that proximal regions of the colon act mainly as storage sites for ileal effluents and in this location, absorption of excess fluid and mixing is the main function. In contrast, peristaltic contractions and relaxations are needed in the distal colon in order to propel dehydrated faeces towards the rectum and therefore, purinergic transient relaxations may predominate in this area.

To test this hypothesis we used murine colon divided in three sections: proximal, mid and distal colon.

We performed identical protocols to the ones described above and obtained the results shown in Tables 3 and 4.

Indeed, purinergic neurotransmission increased while nitrergic responses decreased proximo-distally. Mechanical recordings were in accordance with the recorded electrophysiological responses ((Figure 5, Image 2).

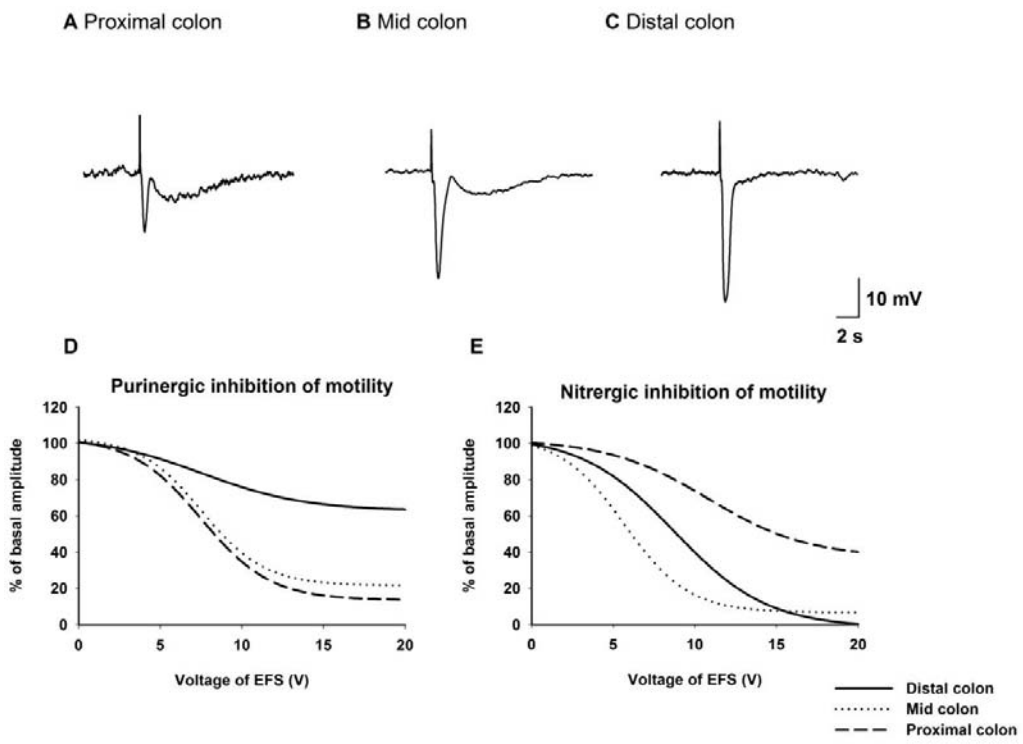

Figure 5. Inverse gradient of purinergic and nitrergic co-transmission along the colon. Purinergic neurotransmission increases proximo-distally while nitrergic neurotransmission predominates in the proximal and mid colon. Electrophysiological (A,B,C) and mechanical recordings (D,E).

		Purinergic response		
		Proximal colon	**Mid colon**	**Distal colon**
Response to increasing voltages of EFS	***IJPf $_{MAX}$***	-15.0 ± 1.0 mV	-28.3 ± 0.8 mV	-32.8 ± 1.4 mV
	Vf50	17.8 ± 1.4 V	19.1 ± 0.5 V	17.4 ± 0.9 V
	Hill slope(f)	-0.13 ± 0.05	-0.13 ± 0.02	-0.12 ± 0.03
Response to increasing frequencies of EFS	***k (f)***	1.8 ± 0.7 s	1.2 ± 0.1 s	2.3 ± 0.5 s
	IJPf$_{\infty}$	-5.1 ± 0.8 mV	-7.6 ± 1.0 mV	-10.3 ± 1.3 mV

Table 3. Experimental values of purinergic neurotransmission in the murine proximal, mid and distal colon.

		Nitrergic response		
		Proximal colon	**Mid colon**	**Distal colon**
	IJPs $_{MAX}$	-11.4 ± 1.1 mV	-9.1 ± 0.4 mV	-1.8 ± 0.3 mV
Response to increasing voltages of EFS	***Vs50***	16.5 ± 2.5 V	13.9 ± 1.1 V	16.5 ± 4.7 V
	Hill slope (s)	-0.11 ± 0.06	-0.16 ± 0.05	-0.07 ± 0.06 V
Response to increasing frequencies of EFS	***k (s)***	0.6 ± 0.2 s	0.6 ± 0.2 s	1.0 ± 0.5 s
	IJPs$_{\infty}$	-28.9 ± 1.8 mV	-21.9 ± 1.7 mV	-9.3 ± 1.3 mV

Table 4. Experimental values of purinergic neurotransmission in the murine proximal, mid and distal colon.

Pre-junctional vs. Post-junctional reasons for the inverse gradient

The differences between regions could be explained by two different reasons: 1-Prejunctional reasons: Amount of neurotransmitter being released in each site or 2-Post-

junctional reasons: Different number of post-junctional cells/receptors receiving the same input. Concentration-response curves with the P2Y agonist ADPβs showed that at least one of the reasons for the purinergic proximo-distal increase is post-junctional as the distal colon responded more to the agonist. This could be due to a superior expression of $P2Y_1$ receptors in this colonic area or more post-junctional cells (either PDGFR+ or SMCs) responding to the neurotransmitter. However, a lower expression of nNOS positive neurons has been described in the rat distal colon (Takahashi & Owyang, 1998), and therefore, according to a co-transmission process, also less purinergic neurons are present in this colonic region. Quantification of the receptor or the post-junctional cells along the colon should be performed to elucidate this issue.

On the other hand, no differences in the response to the NO donor NaNP were observed between the three colonic regions indicating that pre-junctional reasons may account for the differences in nitrergic responses. As mentioned, it has been observed that the proximal and mid colon display a higher amount of nNOS positive neurons (Takahashi & Owyang, 1998). On the other hand, post-junctional reasons cannot be ruled out since studies performed in the rat colon also show a decreased sensitivity to NO (Takahashi & Owyang, 1998) and a lower population of ICC in the distal colon (Ward *et al.*, 2002).

Neural inhibitory tone

An inhibitory neural tone, i.e., a "spontaneous" release of inhibitory neurotransmitters without neural stimulation, is often observed in the colon. It is unmasked by the addition of tetrodotoxin (TTX), which increases spontaneous motility.

NO is the main neurotransmitter responsible for this neural inhibitory tone as blockage of nitrergic neurotransmission has similar effects to TTX. Purinergic neurotransmission, in contrast, generates spontaneous IJP (Gil *et al.*, 2010). These studies were performed in the mid colon and in this work, we wanted to test how the inhibitory tone varied along the colon.

Interestingly, the inhibitory neural tone in each region was in accordance with its predominant neural pathway. While the proximal and mid colon displayed an important nitrergic tone, the distal colon had an MRS2500 sensitive tone, indicative of a purinergic neural tone. Still, the tone observed in the distal colon was of a much lower degree than the nitrergic tone observed in proximal regions. This result is again in concordance with the main physiological role of each colonic region. An inhibitory neural tone is necessary to store colonic contents while enhancing absorption and it therefore needs to be major in proximal regions. We believe that in vitro, the stretching of the strips may emulate the presence of luminal contents and therefore the need of a sustained relaxation.

Transwall gradient

Purinergic and nitrergic responses not only varied along the colon, but also across the muscle layer. The colonic wall displays a transwall gradient of the RMP in which cells near the ICC-SM are more hyperpolarized than the SMCs near the ICC-MY (Figure 6). The cause of this phenomenon has been attributed to the synthesis of CO by submucosal neurons (Sha *et al.*, 2010) or to the own presence of ICC-SM that hyperpolarise surrounding SMCs. The consequence of this fact is that both inhibitory

responses in SMCs near the SM are of lower amplitude due to the proximity of their RMP to the equilibrium potential of potassium, making this area more difficult to hyperpolarize (Image 2).

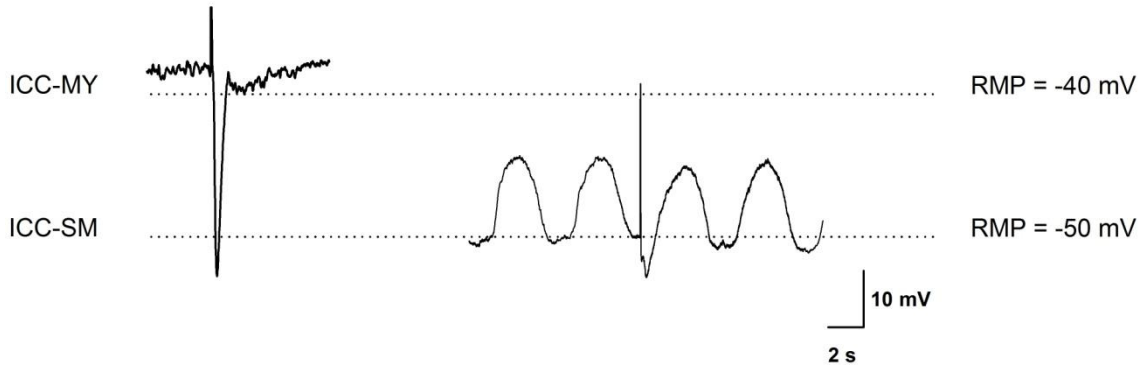

Figure 6. The RMP in muscle cells near the ICC-MY is around -40 mV while in cells near the ICC-SM, where slow waves can be recorded, it oscillates around -50 mV. Consequently, inhibitory junction potentials near the ICC-MY are of greater amplitude.

In conclusion, purinergic and nitrergic neurotransmission show an inverse gradient along the colon. In accordance to the physiological function of each colonic area, nitrergic neurotransmission, responsible for sustained relaxations, predominates in the more proximal regions while purinergic neurotransmission, essential for phasic relaxations, is dominant in the distal regions of the gastrointestinal tract. Moreover, the amplitude of inhibitory responses also varies across the colonic wall due to the presence of a transwall gradient of the RMP. These differences should be taken into account when evaluating inhibitory neurotransmission in colonic samples.

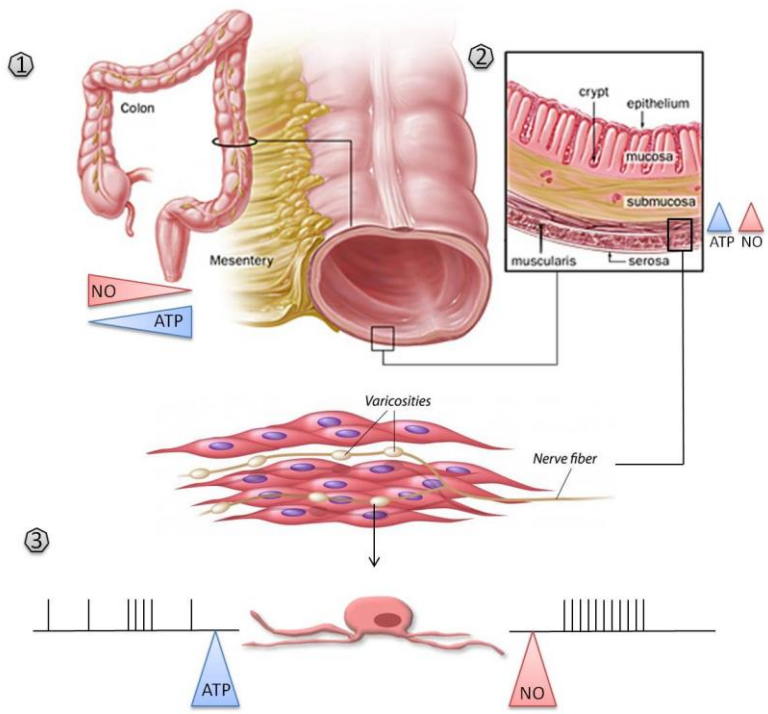

Image 2. Purinergic and nitrergic responses are determined by three factors: 1.- the colonic region, 2.- the RMP of the SMC and 3.- the frequency of neuronal firing.

Effect of inhibitory co-transmission on each pacemaker

The colon displays spontaneous contractions that have their basis on two pacemakers originated in two different networks of ICCs. The ICC-SM generates rhythmic depolarizations known as slow waves while the ICC-MY is probably responsible for cyclic depolarizations of a lower frequency and greater amplitude (Pluja *et al.*, 2001). The ionic origin of slow waves is not fully understood but it most probably involves outward currents through chloride channels and calcium entry through voltage-dependent calcium channels. Nifedipine, an L-type calcium channel blocker, has no effect on slow waves but inhibits cyclic depolarizations (Figure 7). Therefore, it is known that L-type calcium channels are involved in the generation of cyclic depolarizations but not in the genesis of slow waves (Table 5).

L-type calcium channels remain closed at membrane potentials beneath -40 mV. When the threshold is reached, L-type calcium channels open producing calcium inward currents that lead to contraction. Therefore, if the increasing phase of the pacemaker reaches -40 mV, calcium entry through L-type calcium channels evokes contractions at an equivalent frequency. Taking into account that L-type calcium channels participate in the origination of cyclic depolarizations, whenever this pacemaker is displayed, it will always lead to a contraction while the slow wave pacemaker may be present without reaching the threshold for the generation of contractions.

When performing mechanical recordings of colonics strips that preserve both the ICC-SM and ICC-MY, we can observe rhythmic phasic contractions at a high frequency and cyclic contractions of a lower frequency but greater amplitude. These contractions are the mechanical consequence of slow waves and cyclic depolarizations respectively.

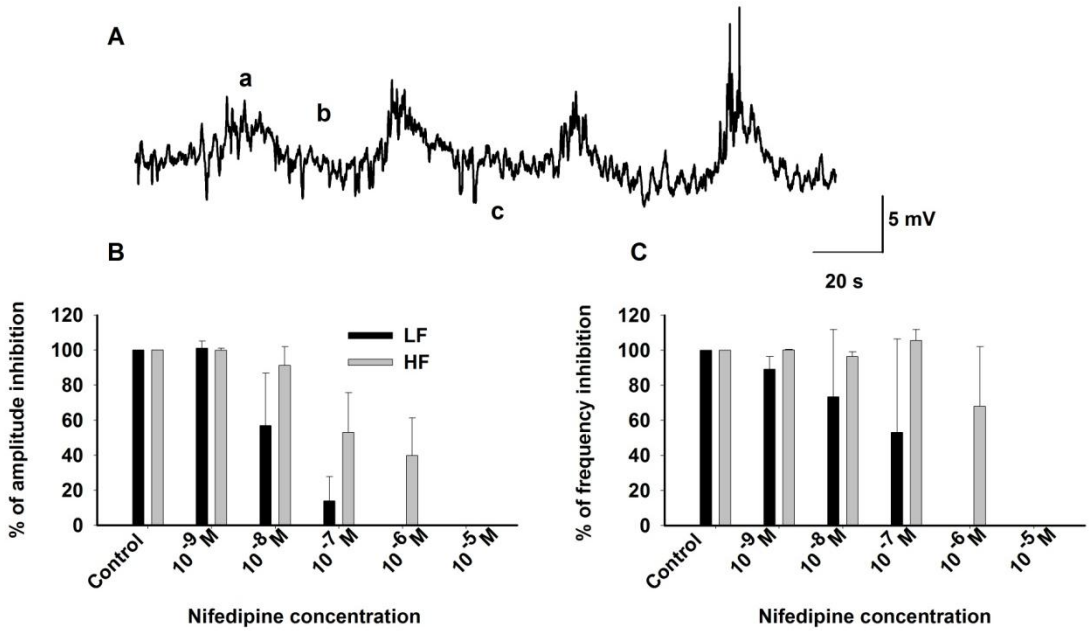

Figure 7. (A) Electrophysiological recording of (a) cyclic depolarizations, (b) slow waves and spontaneous IJP (c). B and C show the lower sensitivity of slow waves associated contractions (HF, grey) to nifedipine compared to the contractions associated to cyclic depolarizations (LF,black).

Once established the role of both ATP and NO in colonic relaxation, our next aim was to *determine the effect of each neurotransmitter on each pacemaker*, as they are known to have a different ionic origin.

Purinergic and nitrergic pathways were tested together and isolated by incubating colonic preparations with ODQ, a GC blocker, and MRS2500 respectively. Low frequency contractions appeared to be very sensitive to hyperpolarisation as they rapidly disappeared with inhibitory neurotransmission due to their dependence on L-type calcium channels. High frequency contractions, in contrast, resisted at high voltages of EFS. This result was consistent with the observations made using the microelectrode technique, where slow waves, instead of disappearing with inhibitory neurotransmission, increased their amplitude when the membrane potential was decreased in order to reach the threshold of opening of L-type calcium channels (Image 3) and cause a contraction.

In this interaction of inhibitory neurotransmission with pacemaker activity, the transwall gradient has to be taken into account. SMCs near the ICC that generate slow waves have a more negative RMP than SMC next to the network of ICCs responsible for cyclic depolarizations. This allows the ICC-MY pacemaker to develop as it needs the opening of L-type calcium channels while slow waves can occur at lower RMP.

In conclusion, the dependency of cyclic depolarizations on L-type calcium channels makes this pacemaker sensitive to hyperpolarisation as when inhibitory neurons release purines and NO, the membrane potential of smooth muscle decreases producing the closing of this type of channels. In contrast, the origin of slow waves is independent of L-type calcium channels and hyperpolariztation increases the slow wave amplitude with no effect on the frequency. In consequence, high frequency contractions can be maintained as the smooth muscle potential still reaches the -40 mV needed to open L-type calcium channels.

Pacemaker	**Nature of the phenomenom**	**Nomenclature**	**Frequency (c.p.m.)**	**Amplitude (g)**	**Nifedipine sensitivity**	**Inhibitory neurotransmis-sion sensitivity**
Low frequency pacemaker	Electrical	**Cyclic depolaritzations**	0.8 ± 0.2 &	9.3 ± 1.6 &	sensitive	sensitive
	Mechanical	**Low-frequency contractions**	0.9 ± 0.1.	4.3 ± 0.5	sensitive*	sensitive
High frequency pacemaker	Electrical	**Slow waves**	10.3 ± 0.5	10.1 ± 2.0	insensitive	insensitive
	Mechanical	**High-frequency contractions**	14.7 ± 0.3	0.25 ± 0.04	sensitive **	sensitive

Table 5. Myogenic spontaneous contractions. Frequency, amplitude, nifedipine and IJP-sensitivity of the two pacemakers displayed by the rat colon in vitro. *(0.01µM-0.1µM) **(1µM-10µM). & According to Pluja et al., 2001 (Pluja *et al.*, 2001)

Origin of the different colonic motor patterns

The last aim of this work was to establish the electrophysiological basis of the different motility patterns recorded in vivo while comparing them with the myogenic motor patterns observed in vitro.

To extrapolate in vitro to in vivo data, it has to be taken into account that when we use colonic strips, we have disrupted enteric neural circuitry that may have a primordial role in the generation of in vivo colonic motor patterns. Besides, we rule out the regulation developed by the parasympathetic and sympathetic branches of the autonomic nervous system.

Circularly-oriented strips from both the rat and human colon display three kind of spontaneous contractions patterns: 1.- High-frequency contractions at an equivalent frequency to slow waves coming from the ICC-SM (Rae *et al.*, 1998;Pluja *et al.*, 2001), 2.- Low-frequency contractions at an equivalent frequency to cyclic depolarizations likely originated in the ICC-MY (Pluja *et al.*, 2001) and 3.- a wax and wane of slow waves and high frequency contractions amplitude (Table 6) (Image 3).

Wax and wane motor pattern

We analyzed the origin of the slow wave wax and wane pattern. A previous publication had shown that in the murine small intestine, this wax and wane of slow waves was associated to segmentation. This study revealed that the pacemaker generated by ICC-MY which, in contrast to the colon, in the small intestine is responsible for slow waves, was modulated by a pacemaker of a lower frequency displayed by the ICC of the deep muscular plexus (Huizinga *et al.*, 2014).

A frequency analysis of our experimental data revealed that the wax and wane of high frequency contractions in both the rat and human colon occurred at the same frequency than low frequency contractions. In fact, low frequency contractions never appeared when the pattern of wax and wane was observed and the wax and wane pattern commonly evolved to a pattern of low frequency contractions superimposed to high frequency contractions.

As observed in the previous section, slow waves increase their amplitude with mild hyperpolarisations while depolarization reduces their amplitude (Tomita, 1978;Jimenez *et al.*, 1999). Indeed, during the wax phase, where the RMP is low, the amplitude of slow waves is high while during the wane phase, the RMP increases and the amplitude of the slow wave decreases.

The coinciding frequencies and the electrophysiological behaviour of slow waves made us conclude that, as observed in the murine small intestine, the low frequency pacemaker generated by the ICC-MY is able to modulate the amplitude of slow waves in the colon.

Correlation of in vitro contractility patterns with in vivo motility

After describing the possible origin of the "wax and wane" motor pattern, we compiled the motility patterns observed in vivo to correlate them with the three contractility patterns observed in in vitro conditions (Table 6).

High frequency contractions have already been clearly associated to in vivo ripples that are displayed at an equivalent frequency to slow waves in the colon (Taylor *et al.*, 1975;Latimer *et al.*, 1981;Narducci *et al.*, 1987;Ford *et al.*, 1995;Dinning *et al.*, 2014). Single motor patterns observed in vivo display very similar features to cyclic myogenic motor patterns indicating that the time course of these contractions is very likely to be orchestrated by the same network of ICCs. Their only difference is that they appear as isolated pressure events. One possibility is that they are the consequence of the wax and wane of slow waves, i.e. that the contractions with the same particularities than ripples only occur during the wax phase, giving raise to isolated trains of contractions. The differences in direction, extent, velocity, frequency or amplitude of the ICC-SM based contractions are due to the coordination exerted by neural circuitries that are in turn influenced by the luminal content and extrinsic innervation.

Propulsive contractions at an equivalent frequency to the low frequency contractions are recorded in vitro in colonic large segments of different species including the human colon (Spencer *et al.*, 2012;Costa *et al.*, 2013). Although they are inhibited after neural blockade and therefore, for a long time classified as neurogenic, addition of carbachol is able to restore these contractions in some species (Huizinga *et al.*, 2011;Costa *et al.*, 2013). Thus, excitatory neural inputs to the ICC-MY (Faussone-Pellegrini *et al.*, 1990;Bayguinov *et al.*, 2010) are probably required to trigger the appearance of the low-frequency pacemaker in vivo but they can afterwards be maintained myogenically. The stretching of the strip performed in vitro may be the substitute trigger of low frequency contractions (Huizinga *et al.*, 2011). Activation of

ascending neural pathways by particular luminal contents may end up enhancing the low frequency pacemaker and generating powerful propulsive contractions such as the high-amplitude propagating contractions (HAPCs) recorded with high-resolution manometry (Dinning *et al.*, 2014). In fact, HAPCs display the same pharmacological characteristics than low frequency contractions. They can be activated by mechanical or chemical stimuli and cannot be blocked by neural blockers such as lignocaine once initiated (Hardcastle & Mann, 1968) (Image 3). In consequence, we hypothesize that cyclic depolarizations are the electrophysiological basis of HAPCs.

In summary, we believe that in the generation of motor patterns, ICCs settle the guidelines which can be modified by enteric neural programs. ICC-MY cyclic depolarizations generating low frequency contractions may be the electrophysiological basis of the propulsive contractions observed in experimental animals and humans. The wax and wane of high frequency contractions is due to the modulation of ICC-SM generated slow waves by the cyclic depolarizations of the ICC-MY. It is associated to a segmentation-like pattern that could be the basis of single motor patterns observed in vivo. Finally, as previously described; ripples are the consequence of slow wave activity in the ICC-SM.

		Motor patterns		
		Propulsive Contractions	**"Segmentation" (Single motor patterns?)**	**Rhythmic propagating contractions**
Rat colon	**Amplitude (g)**	4.3 ± 0.5	Wax/Wane 0.21 ± 0.03/0.09± 0.02	0.25 ± 0.04
	Frequency (c.p.m)	0.9 ± 0.1	1.0 ± 0.1	14.7 ± 0.3
Human colon	**Amplitude (g)**	3.7 ± 2.6	Wax/Wane 2.9 ± 0.5/1.0± 0.2	1.2 ± 0.3
	Frequency (c.p.m)	0.13 ± 0.02	0.3 ± 0.03	2.9 ± 0.3

Table 6. Colonic motor patterns. Amplitude and frequency of the different contractility patterns observed in vitro correlated to their motor patterns in vivo.

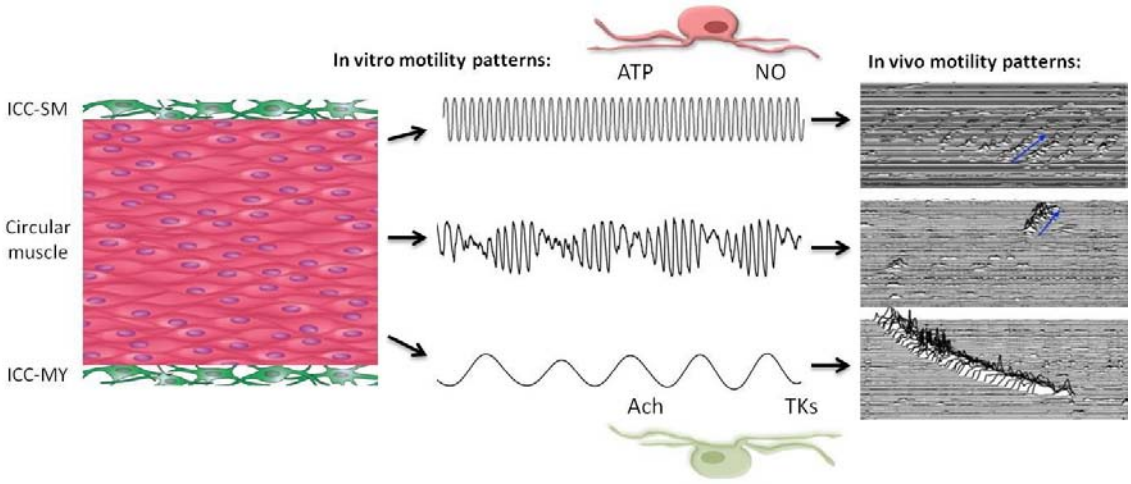

Image 3. The effect that inhibitory neurotransmission has on spontaneous motility is determined by the ionic nature of the pacemaker. These pacemakers are the standards of gastrointestinal motility and are modulated by excitatory and inhibitory neurotransmission. Inhibitory neurotransmission inhibits the low frequency pacemaker and slow waves become dominant. Therefore, in vivo, a motor pattern dominated by ripples will be recorded if slow waves reach the threshold of opening of L-type calcium channels. Excitatory neurotransmission potentiates low frequency contractions and in vivo, propulsive contractions can be observed. Cyclic depolarizations can modulate the amplitude of slow waves (wax and wane) that is the basis of a segmentation-like motor pattern displayed as single motor patterns in vivo.

Final remarks

The use of drugs such as BPTU may modify gastrointestinal motility due to its inhibition of transient relaxations in the digestive tract. This has been observed in

rodents and further experiments should be performed in human samples in order to determine if BPTU also blocks purinergic responses in the human gastrointestinal tract.

Several types of cells contribute to the generation of the different motility patterns needed to develop the varied physiological purposes of the colon. Their interaction contributes to the complexity of motility regulation. ICCs could be defined as "enteric motor pattern generators" which establish the rhythmic basis for the further regulation by enteric neurotransmission. Enteric neurotransmission, in turn, is influenced by the luminal contents and extrinsic innervation. Motor neurons, by releasing excitatory and inhibitory neurotransmitters, enhance or inhibit each pacemaker, which display different sensitivity to neurotransmission. The contribution of each inhibitory neurotransmitter to relaxation depends on the firing frequency of inhibitory motor neurons. This has been observed in both humans and rodents, and even in domestic animals such as horses and therefore, it can be considered a highly conserved mechanism. Moreover, the importance of each neurotransmitter along the gastrointestinal tract may vary according to the main physiological function of each colonic area and responses are graded across the colonic wall. The purinergic-nitrergic inverse gradient along the colon has been observed in the murine colon but should be confirmed in human samples of the different colonic areas.

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CONCLUSIONS

1) Purinergic and nitrergic functional neurotransmission is a highly conserved process of nerve-muscle relaxation in the gastrointestinal tract. It is responsible for relaxation in many areas of the digestive tube and in many species such as humans, rodents and domestic animals.

2) BPTU, a $P2Y_1$ allosteric antagonist, blocks $P2Y_1$ mediated responses in the gastrointestinal tract. This should be taken into account when considering its use as an antithrombotic agent. Allosteric modulation of G-protein coupled receptors is a promising approach for the treatment of colonic motor diseases

3) Different firing frequencies of enteric inhibitory neurons determine the action of one or another co-transmitter. Low firing frequencies or short pulses evoke mainly purinergic responses. Prolonged pulses desensitize the purinergic post-junctional pathway. In contrast, long pulses at high firing frequencies elicit predominant nitrergic responses. Therefore, in order to test the possible impairment of inhibitory neuromuscular transmission in the gastrointestinal tract of human samples or animal models it is important to use an adequate protocol for each neurotransmitter.

4) Purinergic neurotransmission is only able to transiently inhibit spontaneous contractility and is therefore responsible for phasic relaxations. Meanwhile, NO is able to inhibit contractions in a sustained manner, being responsible for tonic relaxations.

5) Purinergic and nitrergic neurotransmission show an inverse gradient along the colon. In accordance to the physiological function of each colonic area, nitrergic neurotransmission, responsible for sustained relaxations, predominates in the more proximal regions while purinergic neurotransmission, essential for phasic relaxations, is dominant in the distal regions of the gastrointestinal tract. These differences should be taken into account when evaluating inhibitory neurotransmission in colonic samples.

6) Cyclic depolarizations are very sensitive to inhibitory neurotransmission due to their dependency on L-type calcium channels. Cyclic depolarizations generating low frequency contractions may be the electrophysiological basis of the propulsive contractions observed in experimental animals and high resolution manometry in humans. In contrast, slow waves which are independent of L-type calcium channels and are responsible for ripples, are insensitive to hyperpolarisation which they respond to with an increase in their amplitude.

7) A transwall gradient exists through the colonic wall. SMCs near the ICC-SM have a more negative RMP than SMCs near the ICC-MY. Therefore, the capacity of inhibitory neurotransmission to hyperpolarize in the site where slow waves are generated is limited due to the proximity of the membrane potential to the equilibrium potential of potassium.

8) The wax and wane of the high frequency contractions developed by slow waves, probably associated to a segmentation-like pattern observed as single motor patterns in vivo, is due to a modulation of slow waves by the cyclic depolarizations originated in the ICC-MY.

9) Motility patterns are the consequence of the interaction between the myogenic rhythm established by ICC and its further regulation by the ENS. Variations in neurotransmission along the colon and across the colonic wall, together with anatomical differences, contribute to the generation of different predominant motor patterns. Therefore, each colonic area can elicit different physiological functions. The ENS is influenced by the luminal content and the autonomic nervous system, adapting colonic motility to each situation.

ANNEX

BPTU, an allosteric antagonist of $P2Y_1$ receptor, blocks nerve mediated inhibitory neuromuscular responses in the gastrointestinal tract of rodents

Neuropharmacology (Accepted, awaiting for publication)

BPTU, an allosteric antagonist of P2Y1 receptor, blocks nerve mediated inhibitory neuromuscular responses in the gastrointestinal tract of rodents.

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Conflict of interest

The authors state no competing interests.

Abstract. $P2Y_1$ receptors mediate nerve mediated purinergic inhibitory junction potentials (IJP) and relaxations in the gastrointestinal (GI) tract in a wide range of species including rodents and humans. A new $P2Y_1$ antagonist, with a non-nucleotide structure, BPTU, has recently been described using X-ray crystallography as the first allosteric G-protein-coupled receptor antagonist located entirely outside of the helical bundle. In this study, we tested its effect on purinergic responses in the gastrointestinal tract of rodents using electrophysiological and myographic techniques. BPTU concentration dependently inhibited purinergic inhibitory junction potentials and inhibition of spontaneous motility induced by electrical field stimulation in the colon of rats (EC_{50}=0.3 μM) and mice (EC_{50}=0.06 μM). Mechanical inhibitory responses were also concentration-dependently blocked in the stomach of both species. Compared to MRS2500, BPTU displays a lower potency. In the rat colon nicotine induced relaxation was also blocked by BPTU. BPTU also blocked the cessation of spontaneous contractility elicited by ADPβS and the $P2Y_1$ agonist MRS2365. We conclude that BPTU is a novel antagonist with different structural and functional properties than nucleotidic antagonists that is able to block the $P2Y_1$ receptor located at the neuromuscular junction of the GI tract.

Keywords: enteric nervous system, purinergic neurotransmission, P2Y1 receptor, allosteric antagonist.

Abbreviations: α,β-meATP, adenosine 5′-triphosphate lithium salt; ADPβS, adenosine 5′-[β-thio]diphosphate trilitium salt; ATP, *adenosine triphosphate; AUC, area under the curve; BPTU,* 1-(2-(2-(tert-butyl)phenoxy)pyridin-3-yl)-3-(4-(trifluoromethoxy)phenyl)urea; *EFS, electrical field stimulation;* GPCR, G-protein-coupled receptor; ICC, interstitial cells of Cajal; IJP, inhibitory junction potential; L-NNA, Nω-nitro-L-arginine; NaNP, sodium nitroprusside; MRS2365, [[(1*R*,2*R*,3*S*,4*R*,5*S*)-4-[6-Amino-2-(methylthio)-9*H*-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt; MRS2500, (1R,2S,4S,5S)-4-[2-Iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt; MRS2179, 2′-Deoxy-N6-methyl adenosine 3′,5′-diphosphate diammonium salt; MRS2279, (1*R**,2*S**)-4-[2-Chloro-6-(methylamino)-9*H*-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0] hexane-1-methanol dihydrogen phosphate ester diammonium salt; NANC, non-adrenergic non-cholinergic*; NO, nitric oxide; nNOS, neuronal nitric oxide synthase;* PDGRFα+ cells, Fibroblast like cells; RMP, resting membrane potential; SMC, smooth muscle cell; tetrodotoxin (TTX).

1. Introduction

Purines in the gut are involved in several physiological functions including neuromuscular transmission, secretion, visceral sensation, synaptic transmission, immunomodulation and gliotransmission (Antonioli *et al*. 2015; Antonioli *et al*. 2008; Burnstock *et al.* 1976; Christofi *et al*. 2004; Cooke *et al*. 2004; Fang *et al*. 2006; Linan-Rico *et al*. 2015; Malin & Molliver 2010; Wunderlich *et al*. 2008). Purinergic receptors are therefore promising pharmacological targets to treat some of the symptoms derived from gastrointestinal disorders (Ochoa-Cortes *et al*. 2014). ATP or a related purine is released from enteric inhibitory motor neurons and causes transient and prominent smooth muscle relaxations. It is co-transmitted with nitric oxide (NO) which is responsible for sustained relaxations of the gastrointestinal smooth muscle (Mane *et al*. 2014a). Purines exert their action by activating $P2Y_1$ receptors in the gastrointestinal tract of mouse, rat, guinea pig and horses (Grasa *et al*. 2009; Hwang *et al*. 2012; Mane et al. 2015; Wang *et al*. 2007). In humans, $P2Y_1$ receptors mediate relaxation both in the colon and small intestine (Gallego *et al*. 2006; Gallego *et al*. 2014). These conclusions have been possible due to the development of selective $P2Y_1$ antagonists such as MRS2179, MRS2279 and MRS2500 (Boyer *et al*. 2002; Boyer *et al*. 1998; Camaioni *et al*. 1998; Kim *et al*. 2001). The finding was later confirmed by demonstrating that $P2Y_1^{-/-}$ knockout mice have impaired purinergic neuromuscular transmission and delayed colonic transit time (Gallego *et al*. 2012; Gil *et al*. 2013; Hwang *et al*. 2012).

$P2Y_1$ receptors are involved in several physiopathological processes outside the gastrointestinal tract that can be attractive for drug development. They contribute to processes such as platelet aggregation (Cattaneo & Gachet 1999), Ca^{2+} signalling and wave propagation in astrocytes (Fam *et al*. 2000; Neary *et al*. 2003) and vascular inflammation (Zerr *et al*. 2011). $P2Y_1$ together with $P2Y_{12}$ receptors mediate platelet aggregation. Antagonists of both receptors act as antithrombotic agents since their blockade significantly decreases ADP-induced platelet aggregation (Bouman *et al*. 2011). In fact, $P2Y_{12}$ antagonists are widely used as antithrombotic

agents (Cattaneo 2007). $P2Y_1$ receptors are also an attractive target for thrombosis treatment (Gachet 2008) as their blockage effectively reduces arterial thrombosis with only a moderate prolongation of the bleeding time (Hechler *et al*. 2006; Kim *et al*. 2003), offering a safety advantage over $P2Y_{12}$ antagonists.

MRS2500 is a competitive antagonist of the $P2Y_1$ receptor with a nucleotidic structure. Bristol-Myers Squibb have recently developed the phenyl urea derivative 1-(2-(2-(tert-butyl)phenoxy)pyridin-3-yl)-3-(4-(trifluoromethoxy)phenyl)urea (BPTU) that also acts a $P2Y_1$ antagonist with potential interest as an antithrombotic agent. BPTU substantially reduces platelet aggregation with a minimal effect on bleeding (Chao *et al*. 2013). A recent paper published in Nature demonstrates, using crystal structures of the $P2Y_1$ receptor, that BPTU is the first selective G-protein-coupled receptor (GPCR) ligand that binds entirely outside of the helical bundle. The binding site between BPTU and the $P2Y_1$ receptor is located on the external receptor interface with the lipid bilayer (Zhang *et al*. 2015). Therefore, BPTU is an allosteric antagonist with a different structure and binding site than previous $P2Y_1$ antagonists that offers new opportunities for drug development.

Structure of P2Y receptors is crucial to identify selective pharmacological tools to modulate functions including those of the nervous system (see for review (von Kügelgen & Hoffmann 2015). We have assessed the effect of BPTU on purinergic neuromuscular transmission in the GI tract of rodents and compared it with MRS2500. As far as we know, this is the first study designed to test BPTU outside its primary target.

2. Materials and methods

2.1 Ethical approval

All procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona.

2.2 Animals and tissue samples

Sixteen CD1 mice of 8 – 18 weeks old (8 males and 8 females) and ten Sprague-Dawley rats aged 12-18 weeks old (5 males and 5 females) were housed under controlled conditions: constant temperature (22 ± 2°C) and humidity (55 ± 10%), 12:12 hour light: dark cycle and *ad libitum* access to water and food. Mice were euthanized by cervical dislocation while rats were euthanized by decapitation. The colon and stomach were removed and placed in carbogenated (95% O_2 and 5% CO_2) Krebs solution. The colon was opened along the mesenteric border and the stomach along the greater curvature. Mucosal and submucosal layers were gently removed from the colon while only the mucosa was dissected from the antrum. 3x5 mm muscle strips were cut in a circular direction.

2.3 Electrophysiological studies

Electrophysiological experiments were performed with colonic rat and mouse strips pinned in a Sylgard®-coated chamber with the circular muscle layer facing upwards. The tissue was continuously perfused with carbogenated Krebs solution at 37±1°C and allowed to equilibrate for 1 h. Phentolamine, propranolol and atropine (all at 1 µM) were added to respectively block α- and β-adrenoceptors and muscarinic receptors and create non-adrenergic non-cholinergic (NANC) conditions. To obtain stable microelectrode impalements, nifedipine 1 µM was added to abolish mechanical activity. L-NNA at 1 mM was also added to the Krebs solution in order to block nitrergic neurotransmission. Circular smooth muscle cells were impaled using glass microelectrodes filled with KCl 3 M (30-60 MΩ of tip resistance). Membrane potential was measured by using a standard Duo 773 electrometer (WPI Inc., Sarasota, FL, USA). Tracings were displayed on an oscilloscope (Racal-Dana Ltd, Windsor, UK) and simultaneously digitalized (100 Hz) with a PowerLab 4/30 system and Chart 5 software for Windows (both from ADInstrument, Castle Hill, NSW, Australia). Inhibitory junction potentials (IJP) were elicited by electrical field stimulation (EFS) using two silver chloride plates placed 1.5 cm apart perpendicular to the longitudinal axis of the preparation. The protocol consisted of single pulse

trains of EFS (0.4 ms pulse duration) at increasing voltages (8, 12, 16, 20, 24, 28, 32, 36, 40 V). The voltage responsible for the supramaximal response was used to elicit single pulses during incubation with BPTU at increasing concentrations ($1x10^{-8}$M, $1x10^{-7}$M, $3x10^{-7}$M, $1x10^{-6}$M and $3x10^{-6}$M). Another train of single pulses at increasing voltages was elicited after the highest dose of BPTU. The amplitude of the IJP (mV) was measured considering it as the difference between the maximal hyperpolarization and the resting membrane potential (RMP).

2.4 Mechanical studies

Muscle strips from the colon and antrum of mouse and rat were mounted in a 10 ml organ bath containing carbogenated Krebs solution in control conditions and in NANC conditions plus L-NNA at 1 mM and maintained at 37 ± 1°C. A tension of 0.5 g and 1 g were applied to mouse tissue and rat tissue respectively and strips were allowed to equilibrate for 1 hour. Mouse vas deferens was isolated of adherent tissues and mounted in the organ bath under 1 g of tension. After this period, antrum and colonic strips displayed spontaneous myogenic phasic activity. Mechanical activity was measured using an isometric force transducer (UF-1 Harvard Apparatus Inc., Holliston, MA, USA) connected to a computer through an amplifier. Data were digitalized (25 Hz) using Data 2001 software (Panlab, Barcelona, Spain) coupled to an A/D converter installed in the computer. The parameters of EFS used for antrum and colonic preparations were pulses at a frequency of 5 Hz, 0.4 ms of duration and 1 min of total duration at 30 V. BPTU and MRS2500 were added at increasing concentrations and incubated for 15 min. At each concentration, a train of EFS of the same characteristics was elicited in order to analyze the antagonist's effect on purinergic relaxation. In other strips, drugs such as nicotine, ADPβS, MRS2365 and α,β-meATP were added to the preparation and added again after incubation for 15 min with antagonists such as BPTU, MRS2500 or A317491. In the vas deferens, the protocol consisted on adding α,β-meATP before and after incubation with MRS2500 and BPTU. Only one protocol was performed in each strip. The area under the curve (AUC) ($g \cdot min^{-1}$) was measured to estimate mechanical activity.

2.5 Drugs and Solutions

The composition of the Krebs solution was (in mM): glucose 10.10, NaCl 115.48, NaHCO3 21.90, KCl 4.61, NaH2PO4 1.14, CaCl2 2.50, and MgSO4 1.16 bubbled with a mixture of 5% CO2-95% O2 (pH 7.4). The following drugs were used: nifedipine, phentolamine, Nω-nitro-L-arginine (L-NNA), adenosine 5′-[β-thio]diphosphate trilitium salt (ADPβS), atropine sulfate, α,β-methylene adenosine 5′-triphosphate lithium salt (α,β-meATP), (−)-Nicotine hydrogen tartrate salt and A317491 sodium salt hydrate (Sigma Chemicals, St. Louis, MO); (1R,2S,4S,5S)-4-[2-Iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0] hexane-1-methanol dihydrogen phosphate ester tetraammonium salt (MRS2500), [[(1*R*,2*R*,3*S*,4*R*,5*S*)-4-[6-Amino-2-(methylthio)-9*H*-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt (MRS2365), propranolol (Tocris, Bristol, UK) and 1-(2-(2-tert-Butylphenoxy)pyridin-3-yl)-3-(4-(trifluoromethoxy)phenyl)urea (BPTU) (Merck Millipore, Darmstadt, Germany). Stock solutions were made by dissolving drugs in distilled water except for nifedipine, which was dissolved in 96% ethanol, A317491, dissolved in DMSO, and L-NNA, which required sonication to be dissolved in Krebs solution. Experiments using vehicles were performed and no effect was observed on spontaneous motility or purinergic neurotransmission.

2.6 Data Analysis and Statistics

Electrophysiological responses in the voltage-response curves were expressed in mV while the progressive reduction of fast IJP (fIJP) amplitude in the dose-response protocol was expressed as a percentage of the supramaximal fIJP amplitude. Non-linear regression was used to fit experimental data to sigmoid curves. BPTU and MRS2500 voltage-response curves were compared using a Two-way ANOVA test followed by a Bonferroni's multiple comparisons test while the effect of increasing doses of BPTU was tested with a Kruskal-Wallis test followed by a Dunn's multiple comparison test. Responses to drugs and EFS in mechanical experiments were expressed as a percentage of the basal AUC of contractions and again non-linear

regression was used to fit experimental data to sigmoid curves. The antagonist's effect was analyzed using Kruskal-Wallis tests followed by a Dunn's multiple comparison test. Statistical analysis was performed with GraphPad Prism 6 for Windows. Data were considered significant when $P<0.05$. N values represent samples from different animals.

3. Results

3.1 BPTU effect on the fIJP

BPTU blocked the supramaximal fIJP in a concentration-dependent manner both in the rat and mouse colon (Kruskal-Wallis test $P<0.0001$ for both). The EC_{50} of BPTU was approximately 0.3 µM and 0.06 µM for the rat and mouse colon respectively (Figure 1) (Table 1).

In the rat colon, higher concentrations of BPTU compared to MRS2500 (EC_{50} ~ 0.02 µM, results published in (Grasa *et al*. 2009)) were needed to block purinergic responses. The hill slope for MRS2500 and BPTU were close to -1. In the mouse colon, in contrast, both drugs display a similar EC_{50} (MRS2500 0.03 µM) but the hill slope was less steep for BPTU than for MRS2500 (Figure 1) (Table 1).

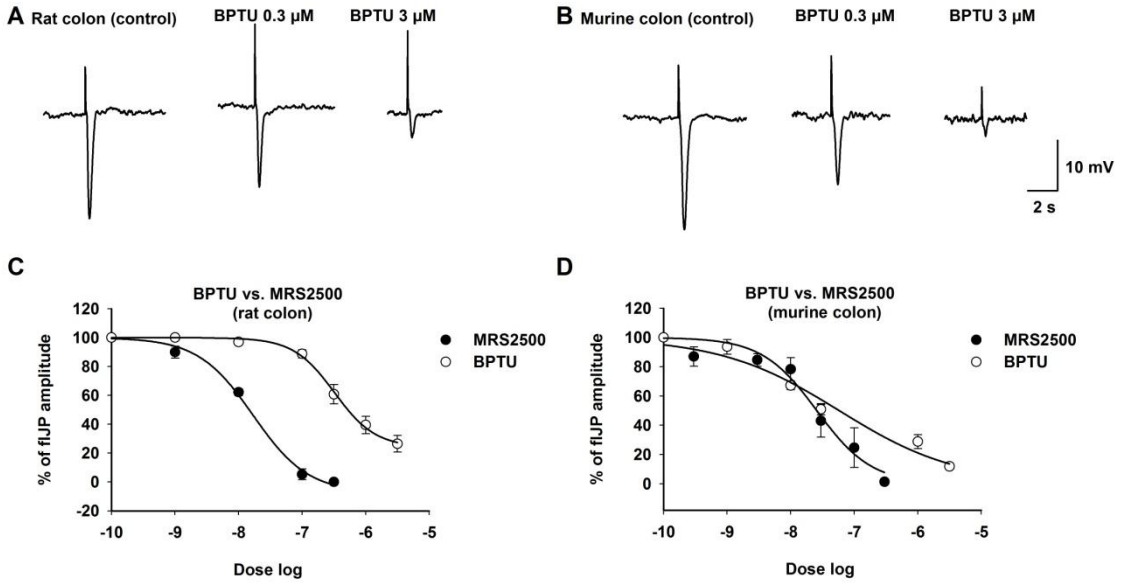

Figure 1. ***BPTU effect on purinergic electrophysiological responses.*** *Reduction of the amplitude of the fIJP at increasing concentrations of BPTU in the rat (A) and murine colon (B). Comparison of sigmoidal concentration-response curves of BPTU and MRS2500 in the rat (C) and murine colon (D).MRS2500 data from mouse and rat colon have been previously published (Grasa et al. 2009; Mane et al. 2016).*

	Rat colon		Mouse colon	
	BPTU	**MRS2500**	**BPTU**	**MRS2500**
LogEC$_{50}$	-6.5 ± 0.1	-7.8 ± 0.1	-7.2 ± 0.1	-7.6 ± 0.1
Hillslope	-1.3 ± 0.3	-1.0 ± 0.2	-0.5 ± 0.04	-1.0 ± 0.2
R^2	0.9	1	0.9	0.8
N	5	4	6	5

Table 1. *Pharmacological data of the blockage of the purinergic electrophysiological response. Conventional sigmoidal concentration-response curves of BPTU and MRS2500 in the rat and murine colon. Data from rat (Grasa et al. 2009) and murine colon (Mane et al. 2016).*

Trains of EFS at increasing voltages caused fIJP that progressively increased their amplitude until reaching a fIJP$_{MAX}$ at high voltages (Mane *et al*. 2014b; Mane *et al*. 2016). BPTU at a concentration of 3 µM strongly reduced the electrophysiological response induced by EFS (Two-way ANOVA rat N=6, P=0.0005, mice N=6, P<0.0001) (Figure 2).

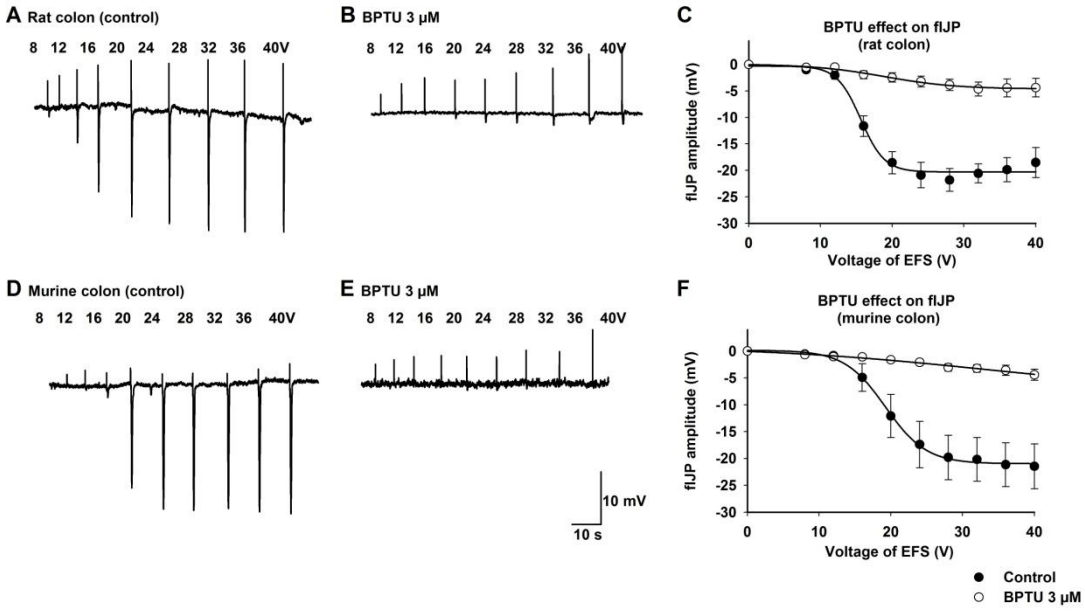

Figure 2. ***Effect of BPTU on fIJP****. Train of EFS at increasing voltages in control conditions (A,D) and after incubation with BPTU 3 µM (B,E) in the rat (top) and murine colon (bottom). Sigmoidal voltage-response curves illustrating the effect of BPTU on fIJP amplitude in the rat (C) and murine colon (F).*

3.2 BPTU effect on the inhibition of spontaneous motility induced by EFS

Rat colonic strips displayed spontaneous rhythmic contractions at a frequency of 0.9 ± 0.06 contractions per minute (c.p.m) and an amplitude of 3.7 ± 0.8 g (N=10). Antrum strips contract at a frequency of 3.5 ± 0.3 c.p.m and an amplitude of 1.6 ± 0.4 g (N=9). Spontaneous contractions in murine colonic strips occur at a frequency of 1.9 ± 0.2 c.p.m and have an amplitude of 1.2 ± 0.8 g (N=6). Strips from the murine antrum displayed 0.3 ± 0.1 g contractions at a frequency of 12.4 ± 3.0 c.p.m (N=5). The presence of spontaneous contractions allows the measurement of a basal AUC (100%) that is then used to calculate the percentage of the remaining AUC when EFS or drugs are applied.

EFS in control conditions diminished the basal AUC to a 2.2 ± 0.8 % (N=5). At the end of the stimulus an off-contraction was recorded. Addition of BPTU at 3 µM had no significant effect in this reduction, i.e., EFS still strongly inhibited spontaneous contractions. Incubation with L-NNA at 1 mM reversed the inhibition of spontaneous contractions elicited by EFS (211.3 ± 29.4 % of basal AUC). Therefore, we used L-NNA at 1 mM from the beginning in order to isolate purinergic neurotransmission.

EFS in NANC conditions and under L-NNA incubation produced a cessation of spontaneous contractions. BPTU blocked the inhibition of spontaneous contractions in a concentration-dependent manner in both the rat and mouse colon (Kruskal-Wallis test $P<0.0001$ for both). The EC_{50} for BPTU was near 0.5 µM and 0.1 µM for the rat and mouse colon respectively. In the antrum, the EC_{50} for BPTU was higher being approximately 9 µM in the rat and 0.3 µM in the murine (Table 2).

The EC_{50} for MRS2500 in the rat colon was obtained from previously published data (Grasa *et al*. 2009) and is around 0.02 µM. In the antrum, the EC_{50} of MRS2500 obtained for blocking purinergic relaxations was approximately 0.3 µM (Figure 3) (Table 2). The % of basal AUC reached after total purinergic blockade was similar between both $P2Y_1$ antagonists. In the rat colon, the AUC in these circumstances was 250% of the spontaneous motility probably due to non-cholinergic excitatory pathways (Figure 3A and C). In contrast, the AUC recorded during EFS was similar to the AUC calculated during spontaneous motility. Accordingly, after cholinergic, nitrergic and purinergic blockade no major excitatory responses were observed in this region (Figure 3B and C).

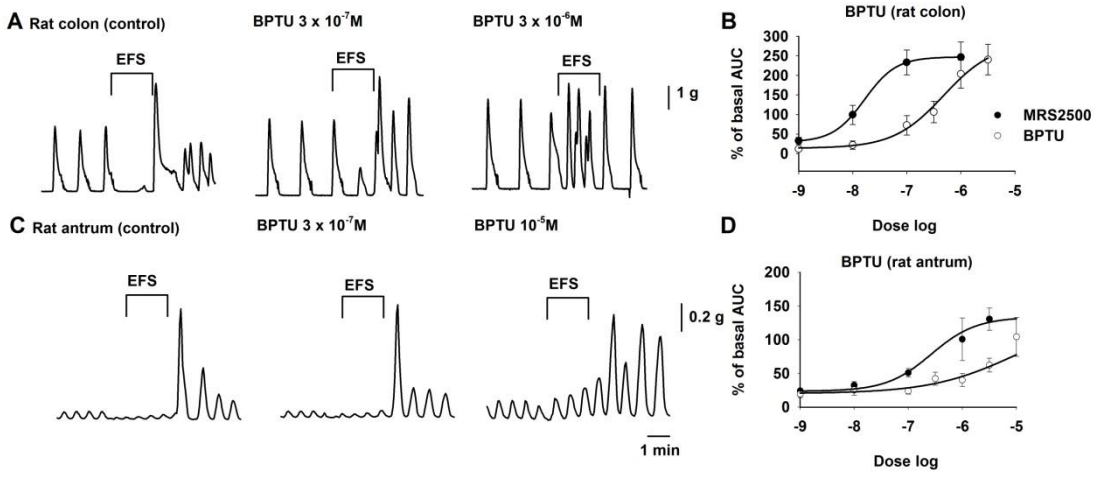

Figure 3. ***BPTU effect on purinergic relaxation in the rat****. 1 min EFS (5 Hz, 30 V and a pulse duration of 0.4 ms) was applied to preparations of colon (top) and antrum (bottom). In control conditions (left) EFS inhibited spontaneous contractions. BPTU at increasing concentrations was added and a reversion of this inhibition could be observed both in the colon (A) and antrum (C). (B and D) Comparison of the potency of BPTU and MRS2500 in the colon and antrum. MRS2500 has a higher potency and the colon is more sensitive to both drugs than the antrum.*

	Rat				**Mouse**	
	Colon		**Antrum**		**Colon**	**Antrum**
	BPTU	**MRS2500**	**BPTU**	**MRS2500**	**BPTU**	**BPTU**
$LogEC_{50}$	-6.3 ± 0.4	-7.8 ± 0.3	-5.0 ± 0.2	-6.6 ± 0.2	-6.9 ± 0.1	-6.6 ± 0.4
Hillslope	0.9 ± 0.6	1.5 ± 0.4	0.6 ± 0.2	1.0 ± 0.4	1.8 ± 0.9	1.1 ± 0.9
R^2	0.7	0.6	0.4	0.5	0.7	0.5
N	6	7	7	5	8	6

Table 2. *Pharmacological data of the blockage of purinergic electromechanical response. Data of the sigmoid curves of BPTU and MRS2500 in preparations from rat (left column) and murine (right column) colon and antrum.*

We tested the reversibility of the drug by incubating mouse colonic tissue with the highest dose of BPTU used, 3 μM (N=5). Washouts were performed every 15 minutes. After 3 hours, response to EFS was not significantly different to the EFS induced response evoked before the washouts, indicating that BPTU action has poor reversibility.

3.3 BPTU effect on nicotine induced responses.

In NANC conditions and under L-NNA incubation, nicotine was also able to cause a transient inhibition of spontaneous contractions to a 57.3 ± 7.0% of basal AUC in the rat colon (N=5) and 44.9 ± 4.8% % of basal AUC in the murine colon (N=4) (Kruskal-Wallis test P=0.0003 for both). 15 minutes incubation with BPTU 3 μM significantly reduced the inhibition produced by the addition of nicotine at 100 μM leaving a 104.1 ± 9.6% of basal AUC in the rat colon and 72.8 ± 5.6% % of basal AUC in the murine colon. Nicotine-evoked responses were also sensitive to MRS2500 at 1 μM (98.6 ± 9.7% of basal AUC) (Figure 4).

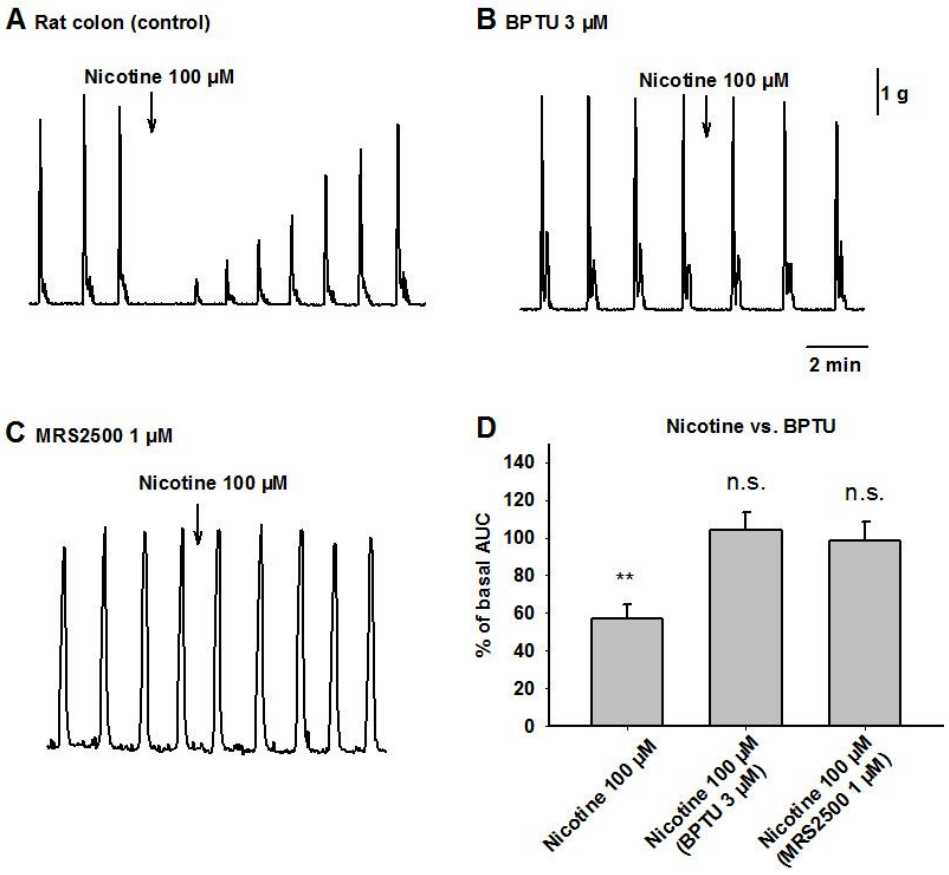

Figure 4. ***Effect of P2Y$_1$ antagonists on nicotine-induced inhibition of spontaneous motility in rat colonic preparations****. Nicotine at 100 µM caused a transient cessation of spontaneous contractions that progressively recovered (A). Under BPTU 3 µM (B) and MRS2500 1 µM (C) incubation, nicotine was not able to inhibit spontaneous motility. (D)Histograms showing the nicotinic response in control and under BPTU and MRS2500 incubation. ** P<0.01, n.s. non significant compared to basal AUC (100%).*

3.4 BPTU effect on P2Y agonists

In the rat colon, addition of the P2Y agonist ADPβS at 10 µM significantly reduced spontaneous contractions to a 43.2 ± 13.4% (N=5) (Kruskal-Wallis test P=0.0002), and this reduction was blocked by 15 min incubation with BPTU at a concentration of 3 µM (93.3 ± 5.1%). Similar results were obtained in the murine colon where ADPβS at 10 µM reduced the AUC of contractions to a 15.8 ± 5.1% (N=4) (Kruskal-Wallis test P<0.0001) and its effect was reversed with BPTU at 3 µM (82.7 ± 3.6%). Addition of MRS2365, a selective P2Y$_1$ agonist, at

a concentration of 5 µM significantly reduced spontaneous contractions to a 21.2 ± 4.8% (N=5) (Kruskal-Wallis test P=0.0002) in the murine colon, and this reduction was blocked by 15 min incubation with BPTU at a concentration of 3 µM (93.1 ± 3.8%). The blockage of the MRS2365-induced response by BPTU at 3 µM also occurred in control conditions (N=5) (10.2 ± 5.5% vs. 86.7 ± 5.0%).

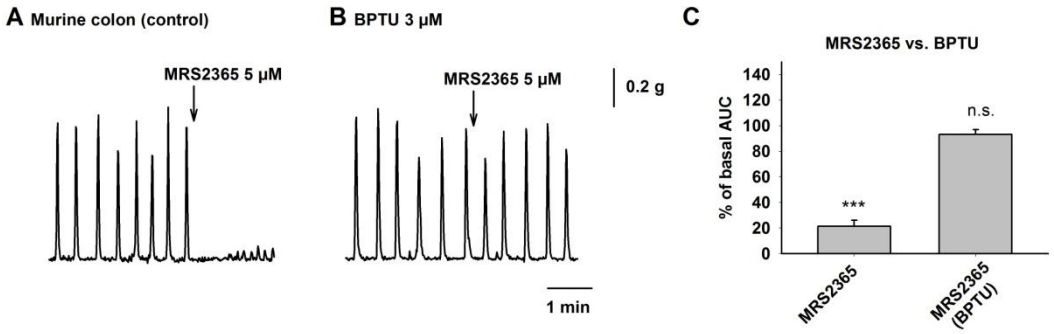

Figure 5. ***BPTU blockage of agonist-induced relaxation in the murine colon****. (A) MRS2365 at 5 µM caused a transient cessation of spontaneous contractions. Under BPTU 3 µM incubation, MRS2365 at 5 µM did not inhibit spontaneous contractions. (C) Histogram showing MRS2365 responses before and after BPTU incubation. *** P<0.001 ,n.s. non significant compared to basal AUC (100%).*

3.5 BPTU effect on P2X preferential agonists

As previously described (Martinez-Cutillas *et al*. 2014) α,β-meATP, a preferential P2X agonist, inhibited the spontaneous contractility of rat colonic strips in a significant manner (N=6) (Kruskal-Wallis test P<0.0001). α,β-meATP at 10 µM also ceases spontaneous contractions in the murine colon (N=5) (Kruskal-Wallis test P<0.0001). In order to test the possible involvement of $P2X_3$ and $P2X_{2/3}$ receptors, we added α,β-meATP before and after incubation with the $P2X_3$ and $P2X_{2/3}$ antagonist A317491 (N=6). After incubating the tissue with the antagonist at 10 µM, α,β-meATP still exerted a similar inhibitory effect (N=6). However, with A317491 at 100 µM, α,β-meATP inhibition of spontaneous contractions was significantly reduced (N=5) (Kruskal-Wallis test P=0.0084). As previously described with the $P2Y_1$

antagonist MRS2500 (Martinez-Cutillas *et al*. 2014), BPTU at 3 μM blocked the relaxation evoked by α,β-meATP (N=6) (Figure 6).

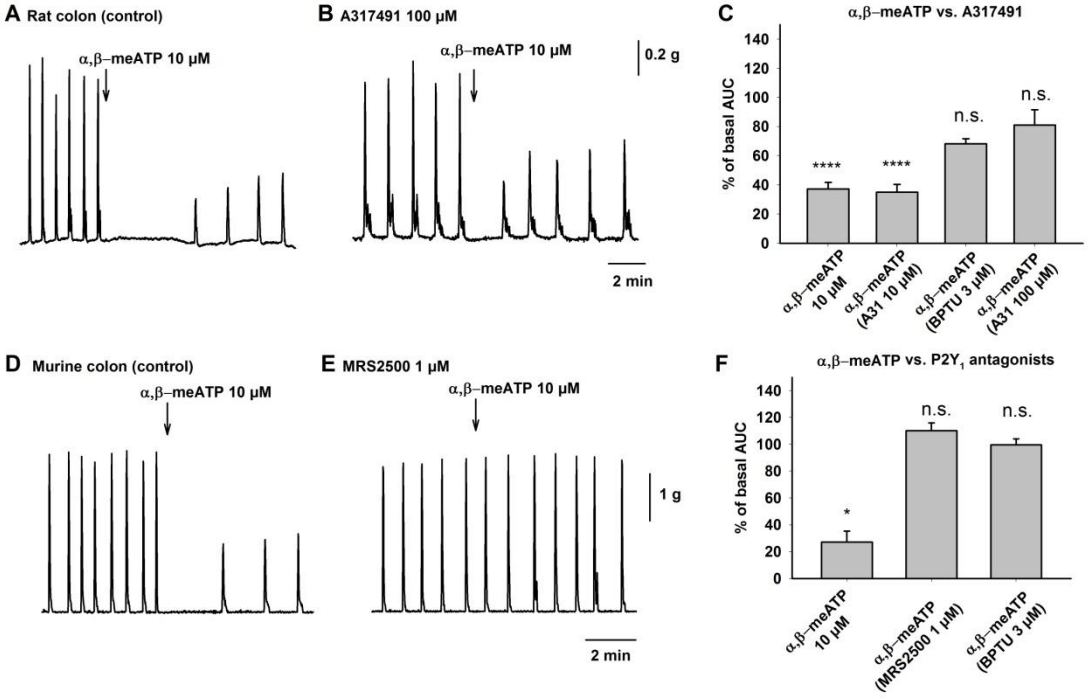

Figure 6. ***Involvement of P2X and P2Y$_1$ receptors in αβ-meATP responses.*** *(A) Addition of αβ-meATP at 10 μM reduced spontaneous contraction in both the rat and murine colon (A and D). (B) Incubation with A317491 (A31 in the figure) at 100 μM reduced αβ-me-ATP responses in the rat colon. (E) MRS2500 at 1 μM reduced αβ-meATP responses in a significant manner in the murine colon. (C) Histogram showing αβ-meATP responses after incubation with A317491 and BPTU in the rat colon. **** P<0.0001, n.s. non significant compared to basal AUC (100%). (F) Histogram showing αβ-meATP responses after incubation with MRS2500 and BPTU in the murine colon. * P<0.05, n.s. non significant compared to basal AUC (100%).*

In order to discard a possible effect of the P2Y$_1$ antagonists on P2X receptors we tested their effect on known αβ-meATP induced responses such as the contractile effect on the vas deferens (Mulryan *et al*. 2000). After a first αβ-meATP induced contraction, we waited for 20 minutes after adding a second dose of αβ-meATP. The ratio between the second and the first contraction was used as the control. Afterwards, we added the P2Y$_1$ antagonists between

contractions and observed if they had any effect on the ratio. No significant differences were observed in the ratio after incubation with neither BPTU (N=6) nor MRS2500 (N=6) (Figure 7).

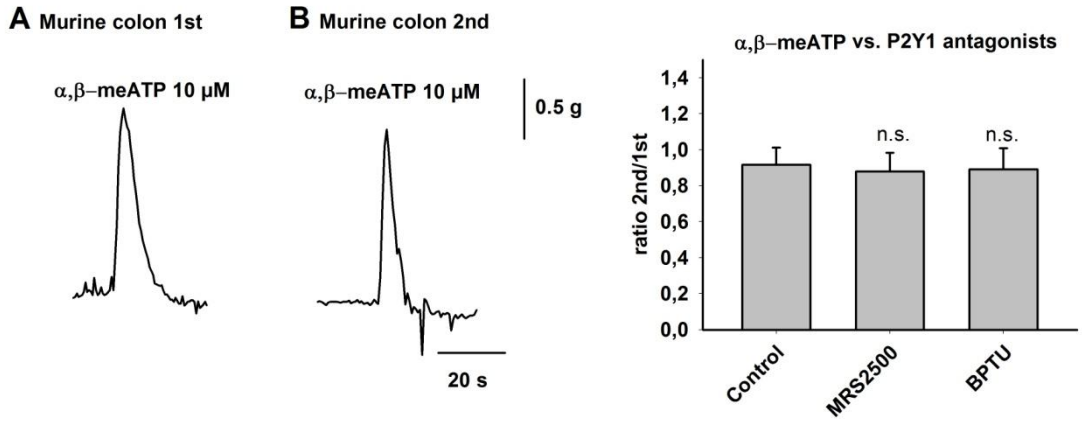

Figure 7. ***BPTU and MRS2500 effect on P2X receptor.*** *(A) αβ-meATP induced contractions of the vas deferens. (B) Second addition of* αβ-meATP *after 20 minutes (B). (C) Neither MRS2500 nor BPTU modified the $2^{nd}/1^{st}$ contraction ratio (One way ANOVA n.s.) n.s. non significant compared to $2^{nd}/1^{st}$ contraction ratio in the absence of antagonists.*

3 Discussion

The identification of the $P2Y_1$ receptor as the receptor responsible for purinergic nerve-mediated relaxation in the GI tract started with MRS2179, the first selective $P2Y_1$ receptor antagonist developed (Boyer *et al*. 1998). MRS2179 blocked nerve-mediated fIJP and mechanical relaxation in the human colon (IC_{50}=1 μM) and small intestine (IC_{50}=0.55 μM) (Gallego *et al*. 2006; Gallego *et al*. 2014). Similar results were obtained in the small intestine of guinea-pigs (Wang *et al*. 2007) and pigs (Gallego *et al*. 2006). However, higher concentrations of MRS2179 (IC_{50}>10 μM) were needed to block purinergic responses in several areas of the GI tract of rodents (Grasa *et al*. 2009; McDonnell *et al*. 2008; Zizzo *et al*. 2007). The development of other purinergic antagonist such as MRS2279 and MRS2500 was essential to demonstrate the crucial role of $P2Y_1$ receptors mediating nerve-mediated relaxation in several areas of the GI tract (Gallego *et al*. 2011; Grasa *et al*. 2009). Lower concentrations of MRS2500 (i.e. IC_{50}=71

nM in the human colon) were needed to block the fIJP (Gallego *et al*. 2011). We have recently published a review where the IC_{50} of the different $P2Y_1$ antagonists from different species and areas of the GI tract are compared (see table 1 in Jimenez et al., 2014). It is important to note that all these compounds are nucleotide derivatives that act as a competitive antagonist of the $P2Y_1$ receptor (Boyer *et al*. 1998; Gao *et al*. 2006). BPTU is a new molecule designed as an antithrombotic agent (Chao *et al*. 2013) displaying two important features 1- it is a phenylurea derivative and 2- it blocks the $P2Y_1$ receptor by binding to a totally different location, outside the helicoidal structure (Zhang *et al*. 2015). In the present manuscript we demonstrate that BPTU concentration-dependently inhibits $P2Y_1$ receptors not only in the surface of platelets but also at the neuromuscular junction in the GI tract. Accordingly, both nucleotide and phenilurea derivatives are able to diffuse and bind to the $P2Y_1$ receptor that is located in this particular domain. The EC_{50} of the sigmoid concentration response curves show that the potency of BPTU is about 1 logarithm lower than MRS2500, being roughly around 1 μM in our experimental conditions. In the rat colon, the rank of potency is then MRS2500>MRS2279>BPTU>MRS2179 both for electrophysiological and mechanical EFS induced responses (Grasa *et al*. 2009). Interestingly, both MRS2500 and BPTU seem less potent in the rat antrum than in the colon but equally potent in the mouse antrum and colon and lower concentrations of BPTU are needed to block inhibitory response in the mouse compared to the rat. We do not have an explanation for these regional and species differences. In the rat, it has previously been described that higher concentrations of MRS2179 were needed to block purinergic response suggesting that the $P2Y_1$ receptor is comparatively less sensitive to these antagonists. Regarding regional differences, one possibility is that in the antrum other purinergic receptors different from $P2Y_1$ are causing nerve mediated relaxation. However, this is probably not true since MRS2500 at 1μM and BPTU at 10μM totally blocked nerve mediated relaxations. Moreover, $P2Y_1$ KO mice lack purinergic neuromuscular transmission in the antrum (Gil *et al*. 2013). A second possibility is that the cell signalling mechanism responsible for nerve mediated purinergic relaxation might not be exactly the same in the colon and antrum. In the colon, nerve-mediated purinergic relaxation can be mediated by PDGFRα+ cells. These

cells express the $P2Y_1$ receptor and respond to $P2Y_1$ agonists with a hyperpolarisation (Kurahashi *et al*. 2014; Kurahashi *et al*. 2012). In a recent manuscript, it has been demonstrated that the sequence of activation involves first a calcium rise in PDGFRα+ cells that activates sK3 channels and then the hyperpolarization is conducted to smooth muscle cells (Baker *et al*. 2015) According to these results, if BPTU is able to block the fIJP, then it is conceivable that it will be acting on $P2Y_1$ receptors located in PDGFRα+ cells. It is unknown if this mechanism recently described in the colon is also present in other areas of the GI tract. In the human stomach, PDGFRα+ cells have been identified (Grover *et al*. 2012); however, the exact function of these cells in gastric motility needs further studies.

In the present manuscript we used different experimental approaches to demonstrate $P2Y_1$ receptor activation. In the organ bath, we applied EFS at 5 Hz to inhibit spontaneous contractions. BPTU, as previously seen with nucleotidic antagonists (Grasa *et al*. 2009), has no effect on the inhibition of spontaneous contractions if NO is present. From this result it can be concluded that 1.- BPTU does not interfere with nitrergic responses and 2.- to study its effect on purinergic responses, tissue must be incubated with L-NNA 1 mM. Both electrophysiological and mechanical purinergic responses were concentration-dependently blocked by BPTU. We used single pulses of EFS at increasing voltage that recruit more inhibitory neurons leading a progressive increase in the fIJP amplitude. The amplitude increase of the fIJP can be fitted to a sigmoid voltage-response curve as previously described (Mane *et al*. 2014a; Mane *et al*. 2014b; Mane *et al*. 2016). A second experimental approach is the use of nicotine. Nicotine causes nerve mediated relaxation in several areas of the GI tract. Nicotine receptors are prejunctionally located and their activation causes the release of inhibitory mediators (Opazo *et al*. 2011; Qian & Jones 1995). This mechanism has been very well characterised in the human lower oesophageal sphincter where NO and $P2Y_1$ receptors contribute to ODQ and apamin sensitive relaxation respectively (Farre *et al*. 2007; Lecea *et al*. 2011; Ruggieri, Sr. *et al*. 2014). It is important to consider these mechanisms as potential pharmacological targets to reduce transient sphincter relaxations that occur during gastroesophageal reflux. Nicotine induced responses

were blocked by both BPTU and MRS2500. Another experimental approach is the use of agonists that act on the $P2Y_1$ receptor. ADPβS, a preferential P2Y agonist, relaxed the colonic smooth muscle. However, ADPβS is not a selective P2Y1 agonist and therefore, we used MRS2365. At a concentration of 5 μM, MRS2365 transiently inhibited spontaneous contractions and this effect was also BPTU sensitive. ADPβS and MRS2365 bind to the $P2Y_1$ receptor at the same place as the natural ligand, which is the same binding site than MRS2500 (Zhang *et al*. 2015). It is important to take into account that the effect of BPTU, in contrast to nucleotidic $P2Y_1$ antagonists, is poorly reversible. This is consistent with the non-competitive binding site and with the nature of the drug.

The expected effect of α,β-meATP on smooth muscle preparations is a contractile response due to its action on P2X receptors, which causes a membrane depolarization and opening of voltage gated calcium channels. However, in gastrointestinal tissue, exogenous addition of α,β-meATP inhibits spontaneous contractions, and the response is both MRS2500 and BPTU sensitive. This is a surprising result apparently not consistent with a direct effect of α,β-meATP on post-junctional $P2Y_1$ receptor (Martinez-Cutillas *et al*. 2014). The blockage of α,β-meATP responses by $P2Y_1$ antagonists could be due to an unselective action of the latter on P2X receptors. In order to characterize their possible interaction with this type of receptors, we performed a protocol that consisted on evaluating the effect of MRS2500 and BPTU on α,β-meATP induced contractions in the vas deferens, which are known to be mainly $P2X_1$ mediated (Mulryan *et al.* 2000). Neither MRS2500 nor BPTU seemed to interfere with α,β-meATP induced contractions and therefore, an action of $P2Y_1$ antagonists on P2X receptors is unlikely the explanation for this result. Another explanation for the blockage of α,β-meATP responses by $P2Y_1$ antagonists could be an activation of pre-junctionally located P2X receptors by α,β-meATP that produces the release of inhibitory neurotransmitters (King 2015; King & Townsend-Nicholson 2008; Linan-Rico *et al*. 2015). In fact, the effect of α,β-meATP was partially blocked by the $P2X_3$ and $P2X_{2/3}$ antagonist A317491. The IC_{50} of A317491 is around 30 nM (Jarvis *et al.* 2002) in cultured cells. To reverse the effect of α,β-meATP, we had to use a concentration of 100 μM at

which A317491 very likely loses selectivity. Nevertheless, studies performed with tissue have commonly used this range of A317491 concentrations (McGarauthy *et al* 2005; King & Townsend-Nicholson 2008). Our results point out that P2X receptors located in inhibitory neurons may participate in the α,β-meATP induced relaxation by activating the release of purines; but the identity of the P2X receptor subtype is still unknown. Further studies should be performed in order to identify the pre-junctional receptor(s) responsible for the action of α,β-meATP.

$P2Y_1$ receptors cause transient relaxations due to the attenuation of purinergic responses. The fIJP runs down in a frequency dependent manner (Mane *et al*. 2014a; Mane *et al*. 2014b; Mane *et al*. 2016). This is consistent with the transient relaxations observed in our experiments (see nicotine response in figure 4A). Post-junction mechanisms involving $P2Y_1$ and its intracellular pathway might be responsible for the rundown of the purinergic inhibitory response. It is unknown if the allosteric binding site occupied by BPTU participates in the rundown mechanism.

In this study we show for the first time the effect of the novel $P2Y_1$ antagonist BPTU on $P2Y_1$-mediated neurotransmission and relaxation in the GI tract. BPTU has proved to block the purinergic receptor efficiently. We used different functional approaches to characterise the effect of BPTU including nerve-mediated responses (EFS, nicotine and α,β-meATP) and a direct activation of the $P2Y_1$ receptor. The effect of BPTU is not restricted to one particular region of the gastrointestinal tract since the effect is present both in the antrum and colon although some regional and species differences were observed. However, species and regional differences in its antagonistic potency raise other concerns about site(s) of action of BPTU in the neural pathways leading to inhibitory neuromuscular transmission.

We still do not have experimental data showing a specific involvement of $P2Y_1$ receptors in GI motor dysfunction although purinergic responses are selectively attenuated in animal models of

intestinal inflammation and $P2Y_1$ KO mice have a delayed colonic transit time (Hwang *et al*. 2012; Roberts *et al*. 2013; Strong *et al*. 2010). Regarding drug development, it is important to consider the possible GI side effects of antithrombotic agents targeting $P2Y_1$ receptors. However, in this case, although the role in GI relaxation of NO differs from purinergic neurotransmission (Mane *et al*. 2014a), the nitrergic pathway may be enough to accomplish an efficient inhibitory motor function. Future studies are needed to test if $P2Y_1$ orthoesteric or allosteric antagonists are useful antithrombotic agents or even potential treatments of GI motor disorders and to establish the possible side effects in the other physiological processes that involve the $P2Y_1$ receptor.

4 Conclusions

BPTU blocks purinergic responses evoked both directly, elicited by $P2Y_1$ agonists, and indirectly, such as the responses evoked by EFS and nicotine. We conclude that this allosteric antagonist is therefore able to reach the $P2Y_1$ receptor located at the neuromuscular junction of the GI tract and to efficiently block purinergic neurotransmission. Such results should be considered in the use of BPTU as an antithrombotic agent. Future tests using BPTU in human GI samples should be performed as it could result a potential treatment for GI motility symptoms.

6. Acknowledgements

This work was funded by the following grant: BFU2009-11118 and 2011 CTP 00032. Noemí Mañé (AP2012-0897) is funded by the Ministerio de Ciencia e Innovación (Spain).

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