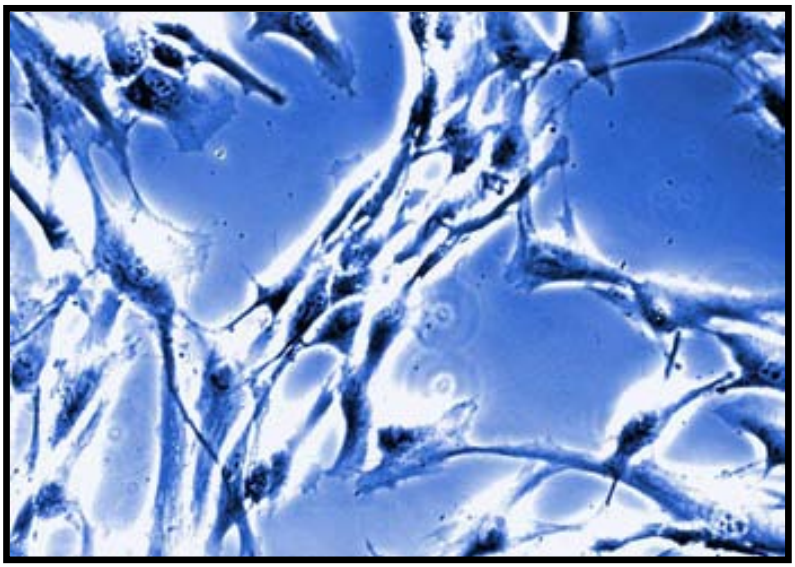
Pharmacological targets mediating colonic smooth muscle relaxation



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Pharmacological targets mediating colonic smooth muscle relaxation

By
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A dissertation submitted in partial fulfillment of the requirements
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We certify that the dissertation entitled "*Pharmacological targets mediating colonic smooth muscle relaxation*" submitted by **MÍRIAM MARTÍNEZ CUTILLAS** in partial fulfilment 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.

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Fem constar que la tesi titulada "*Pharmacological targets mediating colonic smooth muscle relaxation*" presentada per **MÍRIAM MARTÍNEZ CUTILLAS** per optar al Grau de Doctor ha estat realitzada sota la meva supervisió i autoritzo la presentació per realitzar la seva defensa oral.

I, perquè així consti, signem aquest document,

Dra. Maria Teresa Martín Ibáñez,
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“Un genio se hace un 1% de talento y un 99% de trabajo.” **Albert Einstein**

“Permítanme revelarles el secreto que me ha conducido a mi meta. Mi fuerza reside exclusivamente en mi tenacidad.” **Louis Pasteur**

“*I’m a greater believer in luck, and I find the harder I work the more I have of it.”* **Thomas Jefferson**

“All the so-called "secrets of success" will not work unless you do.” **Author unknown**

“It's not that I'm so smart, it's just that I stay with problems longer.” **Albert Einstein**

“I haven’t failed. I’ve just found 10.000 ways that won’t work.” **Thomas Alva Edison**

“Todo parece imposible hasta que se hace.” **Nelson Mandela**

“Las cosas simples son las más extraordinarias y sólo los sabios consiguen verlas”. **Paulo Coelho**

“A ningú costa més que a aquell que molt ho desitja.” **Santiago Ramón y Cajal**

“I’m not in competition with anybody but myself. My goal is to beat my last performance.” **Céline Dion**

“The world is more malleable than you think and it’s waiting for you to hammer it into shape.” **Bono (U2)**

Agraïments

Vaig emprendre el camí de la tesi amb tota la il·lusió del món i disposada a donar el millor de mi. Tot i així, tot i estar a totes, quan es comença un viatge mai sabem els contratemps que ens podem trobar... Durant el doctorat he hagut de lluitar per superar no només incògnites farmacològiques, gràfics impossibles, experiments amb resultats incoherents o una tècnica de calcium imaging que es resistia a funcionar...també m'he hagut d'enfrontar a una batalla només meva i en la qual no tenia opció a abandonar. La lluita va ser molt dura en alguns moments i vaig necessitar tota la meva força de voluntat i valentia. El fet de saber què és patir un problema important de salut m'ha servit per aprendre a valorar moltes coses que abans potser no apreciava suficientment. Pel que fa a la tesi, si hagués de fer balanç, diria que tot i que per mi aconseguir acabar-la no ha estat fàcil i he passat moments molt complicats, ha estat una satisfacció inexplicable i un valuosíssim aprenentatge en molts aspectes. El més important ara és que el meu esforç ha tingut la seva recompensa, i que les adversitats viscudes m'han ensenyat a apreciar molt més les petites coses i a no rendir-me, a ser més forta, a créixer com a persona i a valorar molt més les persones que han estat al meu costat i m'han ajudat a no perdre'm pel camí. Per això vull, de tot cor, agrair a totes les persones que han cregut en mi i m'han fet costat quan més ho necessitava, el seu valuós suport. Estan presents en el meu pensament tots els detalls que heu tingut amb mi i teniu tota la meva gratitud. Sé que sense vosaltres no hauria estat posible arribar fins aquí. M'agradaria també disculpar-me si, sense voler, m'he oblidat algú en aquests agraïments.

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ACRONYMS

2-MeSADP 2-methylthio-adenosine 5'-diphosphate

2-MeSATP 2-methylthio-adenosine 5'-triphosphate

3MST 3-mercaptopyruvate sulfurtransferase

ACh acetylcholine

ADP adenosine 5’-diphosphate

ADP-ribose adenosine 5’-diphosphoribose sodium salt

ADPβS adenosine 5’-[β-thio]diphosphate trilitium salt

ANOVA analysis of variance

AOAA aminooxyacetic acid

ARL67156 6-N,N-diethyl-D-β,γ-dibromomethyleneATP trisodium salt

ATP adenosine 5´-triphosphate

ATPγS adenosine-5-(*γ*-thio)-triphosphate

AUC area under curve

BK large conductance calcium activated K^+ channels

BzATP 2’-3’-O-(4-benzoyl-benzoyl)- adenosine 5´-triphosphate

cAMP cyclic adenosine monophosphate

CBS cystathionine β- synthase

cGMP cyclic guanosine monophosphate

CMC colonic motor complex

CMMC colonic migrating motor complex

CNS central nervous system

CO carbon monoxide

COX cyclooxygenase

CSE cystathionine γ-lyase

EC_{50} half maximal effective concentration

EFS electrical field stimulation

EJP excitatory junction potential

eNOS endothelial nitric oxide synthase

ENS enteric nervous system

GC guanylyl cyclase

GI gastrointestinal

GiC giant contraction

GMC giant migrating contraction

GPCR G protein-coupled receptors

GTP guanosine 5'-triphosphate

H_2S hydrogen sulphide

HA hydroxylamine

HAC high-amplitude contraction

HAPC high-amplitude propagated contraction

HCSMC human cultured colonic smooth muscle cell

HFLA high frequency- low amplitude contraction

HO-1 haem oxigenase-1

HO-2 haem oxigenase-2

IBD inflammatory bowel disease

IBS irritable bowel syndrome

IC_{50} half maximal inhibitory concentration

ICC interstitial cell of Cajal

ICC-IM interstitial cells of Cajal intermixed with smooth muscle fibers

ICC-MP interstitial cells of Cajal associated with the myenteric plexus

ICC-SMP interstitial cells of Cajal associated to the submuscular plexus

IJP inhibitory junction potential

IJPf fast inhibitory junction potential

IJPs slow inhibitory junction potential

iNOS inducible nitric oxide synthase

IP_3 inositol-1,4,5-trisphosphate

IP_3R inositol-1,4,5-trisphosphate receptor operated calcium channels

IPAN intrinsic primary afferent neuron

K_{ATP} ATP-sensitive potassium channels

KO knockout

Kv delayed rectifier K+ channels

LAC low-amplitude contraction

LAPC low amplitude propagated phasic contraction

LDC long distance contraction

LFHA low frequency- high amplitude contraction

L-NNA N^{ω}-nitro-L-arginine

MLC20 20 kDa myosin light chain subunits of myosin

MLCK myosin light chain kinase

MLCP myosin light chain phosphatase

MP myenteric plexus (Auerbach plexus)

n.s. not significant

NaHS sodium hydrogen sulphide

NANC non-adrenergic non-cholinergic

NKA neurokinin A

NKB neurokinin B

nNOS neuronal nitric oxide synthase

NO nitric oxide

NOS nitric oxide synthase

NPTDase ecto-nucleoside triphosphate diposphohydrolase

NT neurotransmitter

OB otilonium bromide

ODQ 1-H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one

PACAP pituitary adenylyl cyclase activating peptide

PAG D,L-propargylglycine

PDGFRα+ cell platelet derived growth factor receptor α-positive cell

PG prostaglandin

PGE_2 prostaglandin E_2

PKA cyclic adenosine monophosphate-dependent protein kinase

PKG cyclic guanosine monophosphate-dependent protein kinase

PLC phospholipase C

RMP resting membrane potential

ROI region of interest

RPC rhythmic phasic contraction

RPMC rhythmic propulsive motor complex

RyR ryanodine receptors

S.E.M. standard error of the mean

sKca small conductance calcium-activated potassium channels

SMC smooth muscle cell

SMP submucosal plexus (Meissner plexus)

SP substance P

TK tachykinin

TRPA1 transient receptor potential cation channel type A1

TRPV1 transient receptor potential cation channel type V1

TTX tetrodotoxin

TXA tromboxane

UDP uridine 5'-diphosphate

UDP-glucose uridine diphosphate glucose

UTP uridine-5'-triphosphate

VDCC voltage dependent calcium channels

VIP vasoactive intestinal polypeptide

WT wild type

β-NAD β-nicotinamide adenine dinucleotide hydrate

ΔF increase in fluorescence

ω-CTX ω-conotoxin

α,β-meATP α,β- methylene adenosine 5´-triphosphate lithium salt

TABLE OF CONTENTS

ABSTRACT

Pharmacological targets mediating colonic smooth muscle relaxation

The principal excitatory neurotransmitters in the colon are acetylcholine and tachykinins, while adenosine triphosphate (ATP) (or related purine) and nitric oxide (NO) are the main inhibitory neurotransmitters causing smooth muscle hyperpolarization and the corresponding relaxation. Recently, hydrogen sulphide (H_2S) has been proposed as an inhibitory mediator. Moreover, prostaglandin E_2 (PGE_2) related pathways might be involved in the control of gastrointestinal contractility. Relaxation of colonic smooth muscle can be reached either by activation of the inhibitory pathways or by blockade of the excitatory ones. The mechanisms of action of several "spasmolytic" mediators capable for inhibiting smooth muscle contractility have been investigated: activation of inhibitory $P2Y_1$ and EP_2/EP_4 G protein-coupled receptors (GPCR), inhibition of the excitatory muscarinic and tachykinergic GPCR and blockade of L-type and T-type voltage dependent calcium channels (VDCC).

Both in the rat and human colon, the non-nitrergic relaxation was inhibited either by MRS2500 or apamin, suggesting that the endogenous purine is activating $P2Y_1$ receptors and leading to the opening of small conductance calcium-activated potassium channels. Several purinergic agonists tested (ATP, ADP, β-NAD and ADP-ribose) inhibited spontaneous contractions but were not antagonized neither by MRS2500 nor apamin. On the contrary, α,β-meATP, a stable analogue of ATP widely considered as a P2X agonist, perfectly mimicked the pharmacological profile of the purinergic transmitter in both species. Activation of EP_2/EP_4 inhibitory GPCR led to relaxation of murine circular colonic smooth muscle. In wild type (WT) animals, PGE_2 and butaprost concentration-dependently inhibited spontaneous contractions and hyperpolarized smooth muscle cells. Combination of both EP_2 (PF-04418948) and EP_4 receptor selective antagonists (L-161,982) was needed to block PGE_2 effects, suggesting activation of both EP_2 and EP_4 receptors. Butaprost inhibitory responses were totally abolished by PF-04418948. In EP_2-knockout mice, no effects were observed after butaprost exogenous addition whereas PGE_2 induced relaxation and hyperpolarization was fully antagonized by L-161,982. In WT animals, EP_2 and EP_4 receptor antagonists caused smooth muscle depolarization and increased spontaneous mechanical activity, suggesting a constitutive release of prostaglandins acting on such receptors. Cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) are two enzymes responsible for H_2S production. In the human colon, combination of the CSE inhibitor D,L-propargylglycine and the CBS inhibitor Aminooxyacetic acid depolarized the smooth muscle and elicited a transient tone increase. The H_2S donor NaHS concentration-dependently inhibited spontaneous contractions in the

presence of tetrodotoxin. This effect was partially reduced by the guanylyl cyclase inhibitor ODQ and by the NO synthases blocker L-NNA. NaHS reversibly blocked neural mediated cholinergic and tachykinergic contractions and also concentration dependently reduced the increase in spontaneous mechanical activity induced by carbachol and neurokinin A (NKA). H_2S might be an endogenous gasomediator regulating human colonic contractility and its inhibitory effect could be due to a possible synergistic effect with NO as well as by an interaction with the cholinergic and tachykinergic pathways. The pharmacological properties of the spasmolytic drug otilonium bromide (OB) have also been investigated. OB concentration-dependently inhibited nifedipine sensitive calcium transients induced by KCl and BayK8644 and also $CaCl_2$ induced contractions in colonic strips. In the presence of nifedipine, OB inhibited atropine-sensitive carbachol-induced and electrical field stimulation-induced muscarinic responses. Moreover, OB inhibited NKA and $CaCl_2$ induced calcium transients. These results demonstrate that OB causes inhibition of L-/T-type VDCC, muscarinic and tachykininergic responses that acting together might explain the pharmacological properties of the compound.

The results presented in the present work suggest that the inhibitory pathways related to $P2Y_1$ and EP_2/EP_4 GPCR and H_2S should be considered as potential pharmacological targets that produce smooth muscle relaxation and therefore could be useful tools to treat spasticity in colonic motor disorders. Further investigation is needed in order to find out their real therapeutic potential.

KEYWORDS: colon, smooth muscle cells, relaxation, inhibitory junction potential, adenosine triphosphate, hydrogen sulphide, otilonium bromide, prostaglandin E_2

Dianes farmacològiques que mitjancen la relaxació de la musculatura llisa del còlon

Els principals neurotransmissors excitatoris al còlon són l'Acetilcolina i les taquiquinines, mentre que l'adenosina trifosfat (ATP) (o purina relacionada) i l'òxid nitric (NO) són els principals neurotransmissors inhibitoris que causen hiperpolarització del múscul llis i la corresponent relaxació. Recentment, el sulfur d'hidrogen (H_2S) ha estat proposat com un mediador inhibitori. A més, les vies relacionades amb la prostaglandina E_2 (PGE_2) han estat relacionades amb el control de la contractilitat gastrointestinal. El següents mecanismes d'acció de diferents mediadors "espasmolítics" han estat investigats: activació dels receptors acoblats a proteïnes G (GPCR) inhibitoris $P2Y_1$ i EP_2/EP_4, inhibició dels GPCR excitatoris muscarinic i taquiquinèrgic i blocatge dels canals de calci voltage depenents (VDCC) de tipus L i T.

En el còlon humà i de rata, la relaxació no nitrèrgica fou inhibida per MRS2500 i apamina, suggerint que la purina endògena activa receptors $P2Y_1$ i canals de potassi activats per calci de baixa conductància. Diferents agonistes purinèrgics (ATP, ADP, β-NAD i ADP-ribosa) van ser capaços d'inhibir la contractilitat però no van ser antagonitzats per MRS2500 ni apamina. En canvi, α,β-meATP, anàleg estable de l'ATP considerat un agonista P2X, va imitar perfectament el perfil farmacològic del neurotransmissor purinèrgic en ambdues espècies. L'activació dels receptors inhibitoris EP_2/EP_4 va produir relaxació de la musculatura llisa circular del còlon de ratolí. En ratolins wild type (WT), PGE_2 i butaprost van causar relaxació i hiperpolarització del múscul llis. La combinació dels antagonistes selectius dels receptors EP_2 (PF-04418948) i EP_4 (L-161,982) fou necessària per blocar els efectes de la PGE_2, suggerint activació d'ambdós receptors. Els efectes inhibitoris de butaprost van ser totalment blocats per PF-04418948. En ratolins knockout pel receptor EP_2 no es va observar cap efecte després de l'addició exògena de butaprost mentre que els efectes de la PGE_2 van ser completament antagonitzats per L-161,982. En animals WT, els antagonistes dels receptors EP_2 i EP_4 van causar despolarització del múscul llis així com un increment en l'activitat mecànica, suggerint una producció constitutiva de prostaglandines activant ambdós receptors. En el còlon humà, la combinació dels inhibidors dels enzims de síntesi de H_2S cistationina β-sintasa i la cistationina γ-liasa van despolaritzar el múscul llis i van produir un increment de to. NaHS, un donador de H_2S, va inhibir les contraccions de forma concentració-depenent. Aquest efecte fou reduït per l'inhibidor de guanilat ciclasa ODQ i per l'inhibidor de la síntesi de NO L-NNA. NaHS va blocar de manera reversible les contraccions colinèrgiques i taquiquinèrgiques neuralment mediades així com l'increment d'activitat mecànica induïda per carbachol i neurokinina A (NKA). El H_2S podria ser un gasomediador endogen capaç de regular la contractilitat de la musculatura llisa

del còlon humà i els seus efectes inhibitoris podrien ser deguts tant a una possible sinèrgia amb NO com a la interacció amb les vies colinèrgiques i taquiquinèrgiques. Hem investigat també les propietats farmacològiques del fàrmac espasmolític bromur d'otiloni (OB). Els increments de calci citoplasmàtic i les contraccions mediades per canals de tipus L sensibles a nifedipina van ser inhibits per OB. En presència de nifedipina, OB va inhibir les respostes muscaríniques sensibles a atropina induïdes per carbacol i estímul elèctric de camp. A més, OB va blocar els increments de calci citoplasmàtic induïts per NKA i $CaCl_2$. Aquests resultats demostren que el OB inhibeix els VDCC de tipus L i T així com les respostes muscaríniques i taquiquinèrgiques, efectes que actuant conjuntament expliquen les seves propietats farmacològiques.

Els resultats presentats en aquest treball suggereixen que les vies inhibitòries relacionades amb els GPCR $P2Y_1$ i EP_2/EP_4 i H_2S podrien ser considerades com potencials dianes farmacològiques pel tractament de l'espasticitat associada a desordres motors colònics.

PARAULES CLAU: còlon, cèl·lules de múscul llis, relaxació, potencial d'unió inhibitori, adenosina trifosfat, sulfur d'hidrogen, bromur d'otiloni, prostaglandina E_2

INTRODUCTION

ANATOMY AND FUNCTION OF THE COLON

The gastrointestinal (GI) tract is responsible for providing the necessary amount of water, electrolytes and nutrients. Opposite to the small intestine, the colon does not play an important role in the absorption of nutrients but is responsible for the absorption of the water and electrolytes for conservation and reduction of fecal mass. The colon is approximately one meter long and is comprised in-between the ileocecal valve and the rectum and in the human species is divided in ascending, transverse, descending and sigmoid colon (Sarna, 2010). From the inside to the outside, the colonic wall is composed by the following circumferentially oriented layers (Figure 1):

The mucosal epithelium, responsible for neuroimmune function, absorption of nutrients and fluids as well as secretion of electrolytes, enzymes and protective mucous. Enteroendocrine cells are spread among this epithelium. The lamina propria and the muscularis mucosa (responsible for the villi movements) are located beneath the epithelial layer.

The next layer is the submucosa, which is constituted by a matrix of collagen fibers containing blood and lymph vessels. The submucosal plexus (Meissner plexus, SMP), one of the two ganglionated plexus of the Enteric nervous system (ENS), is located inside the submucosa and innervates the mucosal epithelial cells and blood vessels.

Underneath the submucosa, circumferentially oriented smooth muscle cells (SMCs) (circular muscle layer) are responsible for the annular contractions. In the small intestine of the majority of the species this muscular layer can be divided in the inner and the outer circular layer that are separated by an aganglionic plexus named the *deep muscular plexus*.

The longitudinal muscle layer consists of fibers that are perpendicularly orientated to the circular layer. In humans, this layer is divided in three taenia coli with a thin longitudinal muscle coat over the rest of the colonic surface. However, in other species, the longitudinal muscle layer is thin and uniform all around the circumference. The myenteric plexus (Auerbach plexus, MP), the second ganglionated plexus of the ENS, is located in-between the circular and the longitudinal muscle layers and contains the neurons responsible for the intrinsic control of GI motility. These neurons are functionally classified as Intrinsic primary afferent neurons (IPANs), interneurons and motorneurons.

Serosal membrane is the last layer of the gut wall and separates the gut from the rest of the organs of the abdominal cavity (Bertrand, 2003; Furness, 2006; Sarna 2010).

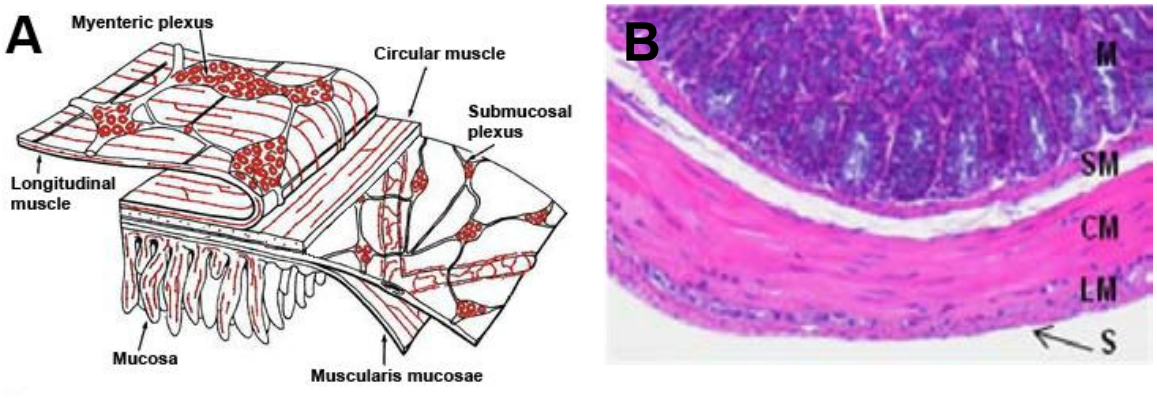

Figure 1. Layers of the colon. (A) Whole-mount drawing adapted from Furness, 2006. (B) Hematoxylin-eosin staining of a colonic cross-section. The different layers can be observed: M mucosa; SM submucosa; CM circular muscle layer; LM longitudinal muscle layer; S serosa. Provided by Dr Anna Domènech.

COLONIC MOTILITY

An adequate GI motility is necessary for an efficient digestion, absorption of nutrients and elimination of waste. It is coordinated by the interplay of several components: circularly and longitudinally oriented SMCs (final effectors) modulated by different control systems, including pacemaker cells (i.e. interstitial cells of Cajal or ICCs) and the ENS (Sanders, 2008). ICCs are electrically coupled to SMCs through gap junctions and are responsible for the initiation of slow wave activity (i.e. cyclic depolarizations recorded in SMCs). The ENS locally modulates mixing and propulsive movements of the small and large intestine, and its activity can be regulated by signals coming from the central nervous system (CNS) or other regions of the GI tract (Kunze & Furness, 1999).

MOTILITY PATTERNS OF THE COLON

Extensive mixing and very slow net distal propulsion is needed to efficiently accomplish colonic motor functions (Sarna, 2010; Bassotti *et al.*, 2005). Colonic contractility can be classified in two main motility patterns: mixing contractions (produce a back and forth movement to mix or turn over the luminal content) or propulsive contractions (larger amplitude and longer duration) (Sarna, 2010). Table 1 summarizes the reported nomenclature for both contractile patterns in the human, mouse and rat colon when different experimental procedures are used:

Table 1. Summary of the nomenclature frequently used to describe colonic motility patterns.

Species	Pattern	Nomenclature	Experimental procedure/References
Human	Propulsive	Colonic propagating sequences (PSs) or propagating contractions: High-amplitude propagated contractions (HAPCs) and Low amplitude propagated phasic contractions (LAPCs).	Manometry (Scott, 2003;Dinning *et al.*, 2010) High-resolution manometry (Dinning *et al.*, 2013)
		Colonic motor complexes (CMCs)	Manometry (Scott, 2003) Organ bath[1] (Spencer *et al.*, 2012)
		Giant migrating contractions (GMCs)	Manometry (Sarna, 2006)
	Mixing	Rhythmic phasic contractions (RPCs)	Organ bath[1] (Rae *et al.*, 1998) Organ bath[1] (Spencer *et al.*, 2012) Manometry (Scott, 2003;Sarna, 2006)
Mouse	Propulsive	Colonic migrating motor complexes (CMMCs)	Organ bath (Fida *et al.*, 1997;Powell *et al.*, 2003)
		High-amplitude contractions (HACs)[3]	Organ bath[1] (Mule *et al.*, 1999)
		Long distance contractions (LDCs)	Organ bath[2] (Chen *et al.*, 2013)
		Giant migrating contractions (GMCs)	Manometry (Gourcerol *et al.*, 2009)
	Mixing	Low-amplitude contractions (LACs)	Organ bath[1] (Mule *et al.*, 1999)
Rat	Propulsive	Giant migrating contractions (GMCs)	Strain gauges (Li *et al.*, 2002)
		Giant contractions (GiCs)[3]	Organ bath[1] (Gonzalez & Sarna, 2001)
		Rhythmic propulsive motor complexes (RPMCs)	Organ bath[2] (Huizinga *et al.*, 2011;Gil *et al.*, 2013b)
		Low frequency-high amplitude contractions (LFHA)[3]	Organ bath[1] (Pluja *et al.*, 2001;Alberti *et al.*, 2005)
	Mixing	Rhythmic phasic contractions (RPCs)	Strain gauges (Li *et al.*, 2002) Organ bath[1] (Gonzalez & Sarna, 2001)
		High frequency-low amplitude contractions (HFLA)	Organ bath[1] (Pluja *et al.*, 2001;Alberti *et al.*, 2005)
		Ripples	Organ bath[2] (Huizinga *et al.*, 2011;Gil *et al.*, 2013b)

([1]) In vitro organ bath experiments in isolated colonic segments: mechanical activity measured by using isometric force transducers.
([2]) In vitro organ bath experiments in isolated colonic segments: video recordings of mechanical activity and analysis performed with Spatio-temporal maps.
([3]) According to the literature, these contractile patterns recorded in vitro may be associated to propulsive events.

In several species, three main types of colonic contractions have been described in vivo: rhythmic phasic contractions (RPCs, dependent of slow waves and responsible for the mixing of the luminal content), giant migrating contractions (GMCs, slow wave independent and propulsive) and tonic contractions (also slow wave independent and responsible for the narrowing of the lumen size facilitating the effectiveness of propulsion and mixing) (Sarna, 2010; Sarna, 2006;Gonzalez & Sarna, 2001).

The amplitude of RPCs generally is not enough to occlude the lumen and consequently have a few or no participation in propagation events. Two subtypes of RPCs have been described in human and canine circular colonic muscle: short-duration and long-duration RPCs. Long-duration RPCs are less frequent but can last over a few minutes and collaborate in the mixing and slow propulsion of feces (Sarna, 2010; Gonzalez & Sarna, 2001).

In humans, GMCs occur randomly and infrequently (about 2-5 times per day) and consist of large-amplitude contractions that occlude the lumen and produce mass movements by fast distal propagation (1 cm/s) over appreciable distances. They can even propagate in the entire length of the colon (Sarna, 2010; Gonzalez & Sarna, 2001). GMCs are related to defecation and to peristalsis since they elicit descending inhibition that suppresses spontaneous contractions and reduces the tone in the distal segment allowing a proper accommodation of the luminal content (Sarna, 2010) (Figure 2). In rats, due to the strong differences in GI physiology and defecation patterns, GMCs occur 20-40 times per hour and are considered the main feature in the motor activity of the rat colon. In rodents GMCs are responsible for the gradual distal pellet propulsion (Sarna, 2010; Li *et al.*, 2002).

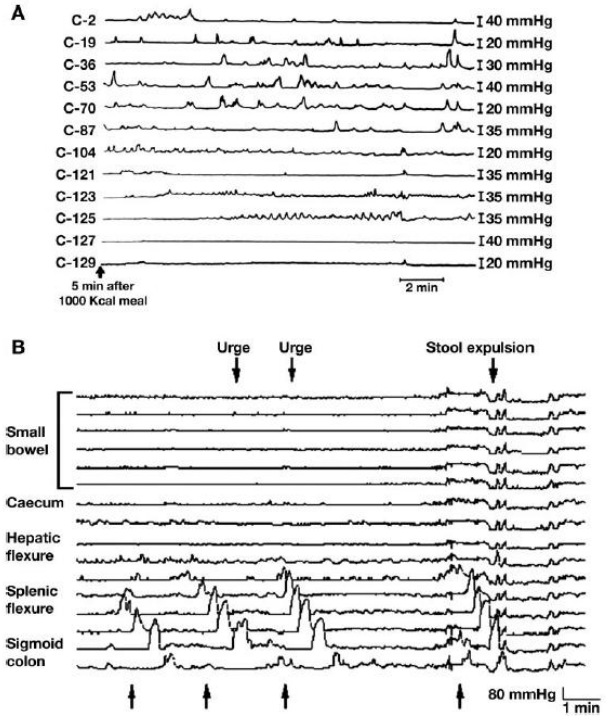

Figure 2. Human colon postprandial manometric recordings. (A) Non-propagating rhythmic phasic contractions (RPCs) found largely in the colon and (B) propagating GMCs, associated to defecation, are represented. The numbers after "C" represent the distance from the manometric tube tip. Adapted from Sarna, 2006.

Video recordings of motility segments have been an interesting way to analyse intrinsic motility patterns in several species including rabbits, guinea-pigs, mouse and rats (Costa *et al.*, 2013). High-frequency ripples are associated with slow wave activity whereas propulsive contractions are associated with nerve-mediated cholinergic inputs (Huizinga *et al.*, 2011).

Although it is not possible to demonstrate propagating events in colonic strips, the contractile patterns observed in vitro might sometimes be correlated to the propulsive or mixing motor patterns observed in the whole colon (Figure 3). For instance, in the mouse colon, low-amplitude contractions (LACs) and high-amplitude contractions (HACs) have been associated to the mixing RPCs and the propulsive colonic migrating motor complexes (CMMCs) respectively (Fida *et al.*, 1997;Mule *et al.*, 1999). In the rat colon, high frequency-low amplitude contractions (HFLA) have been related to mixing events whereas low frequency-high amplitude contractions (LFHA) have been related to propulsion (Gonzalez & Sarna, 2001;Pluja *et al.*, 2001;Alberti *et al.*, 2005). Indeed, the frequency of LFHA is similar (0.6 contractions per min) to that of in vivo recorded GMCs, which have also been associated to giant contractions (GiCs) displayed by isolated colonic strips (Pluja *et al.*, 1999;Gonzalez & Sarna, 2001;Li *et al.*, 2002).

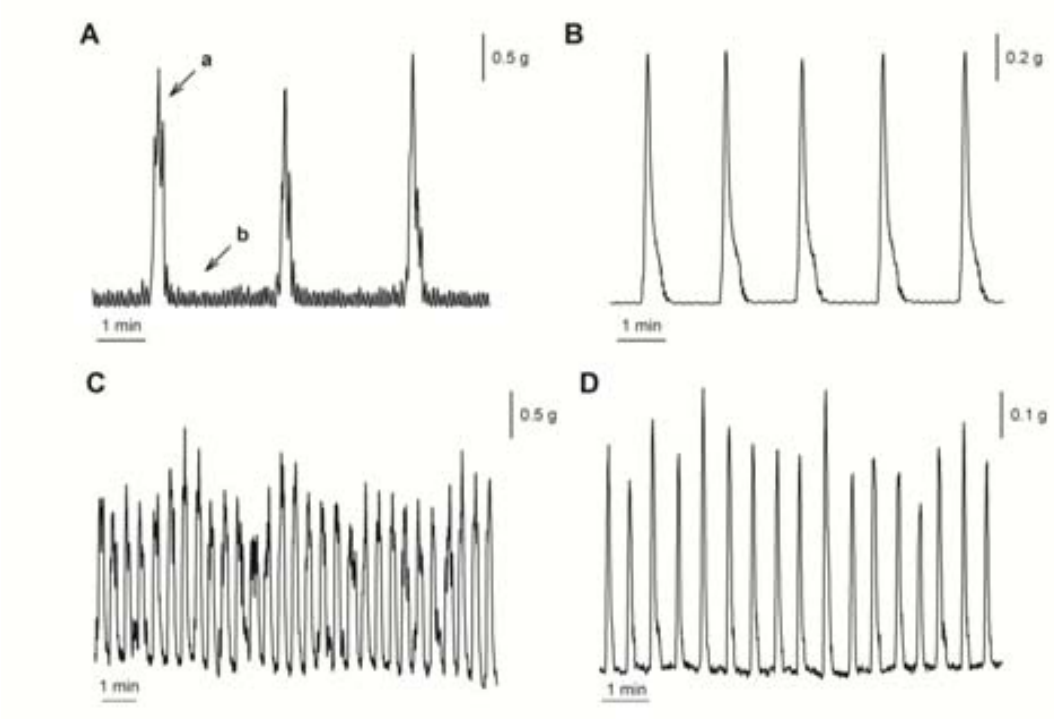

Figure 3. Organ bath recordings showing the myogenic mechanical activity displayed by circularly oriented murine and human colonic strips. (A) Low frequency-high amplitude contractions (LFHA) (a) and High frequency-low amplitude contractions (HFLA) (b) in strips from rat middle colon devoid of mucosa layer. (B) LFHA in strips from rat middle colon devoid of both mucosa and submucosa layers. (C) Rhythmic phasic contractions (RPCs) displayed by human sigmoid colonic strips devoid of mucosa. (D) Mechanical activity displayed by strips from mouse middle colon devoid of both mucosa and submucosa layers (possibly corresponding to the High-amplitude contractions (HACs) described by Mule *et al.*, 1999.

GASTROINTESTINAL MOTILITY: MECHANISMS OF CONTROL

NON-NEURAL CONTROL

Pacemaker activity and electrical activity of smooth muscle cells

In 1899, Bayliss and Starling gave evidence of a gut pacemaker when they found that effective myogenic peristaltic activity occurred even after the blockade of the neural activity (Bayliss & Starling, 1899). A few years later, Santiago Ramón y Cajal described for the first time ICCs as a new type of neural cells (Ramón y Cajal, 1904). ICCs were considered a kind of exception of the neural theory because they were associated in reticular form creating a network of cells. In 1982, Lars Thuneberg proposed that ICCs were the pacemaker cells of the GI tract (Thuneberg, 1982). ICCs are in fact non-muscular cells of mesenchymal origin (Kluppel *et al.*, 1998) that generate spontaneous depolarizations responsible for slow wave activity (Langton *et al.*, 1989). The electrical coupling of ICCs to SMC determines the resting membrane potential (RMP) of SMC-ICC syncitium and it is responsible for the spontaneous electrical activity intrinsic to different smooth muscle types that generate slow waves. Slow wave activity is responsible for excitation-contraction coupling since the corresponding depolarization of SMC membrane increases the open probability of voltage dependent calcium channels (VDCC) and, thus, allows the entry of calcium that leads to SMC contraction (Sanders *et al.*, 2006;Sanders, 2008).

Activation of calcium-activated chloride channels present in ICCs generates inward currents that trigger slow wave activity (Sanders *et al.*, 2012). Studies with knockout (KO) mice have also confirmed the contribution of T-type calcium channels (also named low-voltage-gated dihydropiridine-resistant channels) to the upstroke of the pacemaker potential (Gibbons *et al.*, 2009). T-type calcium channels together with L-type calcium channels (also named high-voltage-gated dihydropiridine-sensitive channels) have been identified both in SMCs and ICCs (Gibbons *et al.*, 2009;Beyder & Farrugia, 2012). Different involvement on slow wave activity has been reported for L-type or T-type calcium channels: blocking L-type currents with nifedipine causes cessation of mechanical activity but does not alter slow wave activity; however, a decrease in frequency and amplitude of slow waves is observed after the blockade of T-type currents in the presence of mibefradil (Dickens *et al.*, 1999;Beyder & Farrugia, 2012).

Immunohistochemistry studies have revealed the distribution of ICCs using the presence of Ano1 and c-kit genes in ICCs (Ward *et al.*, 1994;Huizinga *et al.*, 1995;Alberti *et al.*, 2005;Gomez-Pinilla *et al.*, 2009). In the colon, ICCs are distributed in three plexuses: ICC-SMP

(ICCs located along the submucosal surface of the circular muscle layer); ICC-MP (associated with the myenteric plexus) and ICC-IM (intermixed with smooth muscle fibers). ICC-SMP is the responsible for the onset of slow wave activity in the canine and human colon (Liu & Huizinga, 1993;Smith *et al.*, 1987;Rae *et al.*, 1998). In this way, in the human colon it has been observed that slow waves are greatest in amplitude near the submucosa and their amplitude decreases with distance to submucosal edge (Rae *et al.*, 1998). In the rat colon, two independent pacemakers have been described: one pacemaker constituted by the ICC-SMP that generates the HFLA (associated to slow waves) and another one to the ICC-MP, responsible for the LFHA (associated to cyclic depolarizations) (Pluja *et al.*, 2001).

ICCs might not only generate electrical slow wave activity but also might be responsible for other important functions in the gut such as transduction of motor neuronal inputs coming from the ENS to SMCs, conduction of the stimulus from single neuronal endings to groups of SMCs (amplification of the signal and regulation of the responsiveness to neurotransmitters (NTs)), connection between longitudinal and circular muscle layers and participation in sensory mechanisms as mechanosensation to stretch of GI muscles (Lecci *et al.*, 2002;Sanders *et al.*, 2006;Al-Shboul, 2013).

Several studies have suggested a defective neuromuscular transmission in genetically modified animals with ICC depletion (Ward *et al.*, 2000;Alberti *et al.*, 2007). It has been reported that ICC deficient mice have impaired nitrergic neurotransmission and smooth muscle relaxation (Burns *et al.*, 1996). However, other studies have attributed these findings to the abnormalities associated to the genetic modification which increases smooth muscle excitability rather than to a defective nitrergic neurotransmission (Huizinga *et al.*, 2008).

Smooth muscle cells and calcium signalling

The phosphorilation/dephosphorilation of the 20 kDa myosin light chain subunits of myosin (MLC20) regulates smooth muscle contraction/relaxation (Somlyo & Somlyo, 2003;Sanders *et al.*, 2012). The phosphorilation occurs mainly by calcium-calmodulin dependent actions of a myosin light chain kinase (MLCK); therefore the primary driving force for contraction is the increase in cytoplasmic calcium. Myosin light chain phosphatase (MLCP) balances the phosphorilation of MLC20 and thus allows relaxation (Sanders, 2008;Sanders *et al.*, 2012).

Intracellular calcium is a versatile and complex signalling messenger that regulates many different cellular processes, with great relevancy in contractile tissues as GI smooth

muscle. Although the dynamics of calcium signalling can vary between cell types, the common property is the generation of brief located calcium pulses, also named calcium transients, created by variations of the basic on/off reactions that lead to an increase/decrease of the intracellular calcium levels (Sanders, 2008).

Intracellular calcium increase has two main sources: the external medium and the internal stores. There are many different plasma membrane channels that control the entrance of calcium from the external medium in response to stimuli as depolarization, stretch, extracellular agonists, intracellular agonists or depletion of intracellular calcium stores (Berridge *et al.*, 2003). Nevertheless, voltage dependent L-type calcium channels are the main responsible for calcium entry into SMC. T-type calcium channels also participate in GI motility regulation (Beyder & Farrugia, 2012). The intracellular source of calcium in the muscle is the sarcoplasmic reticulum, where two major receptors are present: inositol-1,4,5-trisphosphate (IP_3) receptor operated calcium channels (IP_3R) and ryanodine receptors (RyR), with a ratio IP_3R: RyR of 10:1 (Sanders, 2008). The proximity between sarcoplasmic reticulum and plasma membrane allows calcium transients to reach high concentrations in microdomains and, thus, to regulate the activity of calcium-sensitive plasma membrane proteins such as small and large conductance calcium-activated potassium channels, chloride channels (less frequent in the GI tract) or non-selective cation channels (Sanders, 2008;Sanders *et al.*, 2012).

IP_3 is generated by Phospholipase C (PLC) that can be activated by different mechanisms such as the activation of G protein-coupled receptors (GPCR). The dynamics of IP_3 production are variable depending on the receptor and the cell that has been activated. Muscarinic receptors, adrenoreceptors, histamine receptors, prostanoid receptors and tachykinin (TK) receptors (NK_1, NK_2 and NK_3) are examples of receptors that, when activated, are able to switch on the GPCR downstream pathway linked to calcium signalling (Berridge *et al.*, 2003).

Electrophysiology of excitation-contraction coupling

Patterned firing of excitatory-inhibitory motor neurons regulates the motor patterns of the GI tract. The access of calcium to the contractile proteins of SMC is controlled by the electrophysiological properties of the syncitium SMC-ICC. SMC have negative RMP determined by the relative permeability of the membrane to the physiological intra and extracellular ionic concentrations, having a dominant permeability to K^+ ions with significant contributions from non-selective cations. The sodium pump has a minor contribution (just several mV) to establish the RMP, which oscillates from -85 to -40 mV depending on the region of the gut. RMP is the

main factor regulating the open probability of L-type calcium channels, and, therefore, controlling SMC excitability. RMP oscillations elicited by slow waves allow the excitation/relaxation coupling of SMC and thus the mixing and movement of luminal contents: excitatory agonists trigger inward currents through non-selective cation channels, depolarize the membrane potential, increase the amplitude of slow waves and allow the activation of voltage-dependent L-type calcium channels that, in turn, generates a contraction (Figure 4). In contrast, inhibitory mediators activate outward currents through potassium channels or suppress tonic inward currents causing hyperpolarization that reduces the amplitude of slow waves, the amount of calcium entry and, consequently, the force of contraction (Sanders, 2000;Sanders, 2008).

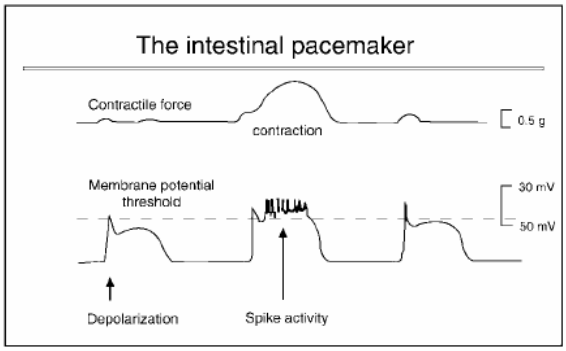

Figure 4. Graphical correlation between the electrophysiology registration of the spiking superimposed to the slow waves (down) and the evoked contraction (up) that happens when the threshold for the opening of calcium channels is reached. Adapted from Hansen, 2003a.

NEURAL CONTROL

Enteric nervous system

The ENS is responsible for the intrinsic innervation of the gut. The ENS is constituted by a huge amount of interconnected neurons (Furness, 2006). According to their function, enteric neurons are classified as:

- Intrinsic primary afferent neurons (IPANs): sensory neurons responding to mucosal receptors.
- Interneurons: responsible for the polarization of neuronal inputs coming from afferent fibers in both oral and aboral directions.
- Motor neurons: responsible for the activation of GI effectors such as SMCs, glands or blood vessels. According to their neurochemical code they can be classified in excitatory or inhibitory motor neurones (Furness, 2000;Sanders, 2008).

The interplay of these functionally different types of neurons permits the mediation of reflexes and motor patterns independently of the inputs from the CNS. However, the CNS can exert an influence on ENS functions through the autonomous nervous system. Both parasympathetic and sympathetic components have aferent (sensitive) and eferent (motor) fibers, which are responsible for the extrinsic control of gut motility. Parasympathetic eferent fibers are cholinergic and their preganglionic neurones are located in the vagus and pelvic nerves. The parasympathetic postganglionic neurones are placed within the ENS ganglia. The sympathetic eferent fibers (mainly noradrenergic) can inhibit the gut motility by interacting with the ENS or directly with the smooth muscle (Hansen, 2003a;Hansen, 2003b).

Propulsive motility is responsible for the transport of the bolus to the anal side of the gut. It is also named peristalsis and is carried out by a complex pattern of neural reflexes that elicit downstream relaxation (descending inhibitory reflex) and upstream contraction of intestinal smooth muscle (ascending excitatory reflex) (Lecci *et al.*, 2002). Peristaltic reflex (Figure 5) is initiated when epitelial sensory transducers, such as enterochromaffin cells secrete serotonin in response to a mucosal stimulus (for instance certain chemicals or intraluminal volume reaching a threshold) (Costa *et al.*, 2000). This phenomenon triggers activation of IPANs that release acetylcholine (ACh) and calcitonin gene-related peptide. IPANs have contacts with interneurons that generate an ongoing polarized peristaltic reflex activity. Interneurons activate excitatory motor neurons causing contraction to the oral side of the stimulus and inhibitory motor neurons causing relaxation to the anal side. A third phase of the peristaltic reflex exists, and it consists of a post-stimulus excitatory response also named “rebound contraction” that follows the previous inhibitory response (Bennett, 1966;Smith *et al.*, 2007;Huizinga & Lammers, 2009;Sanders *et al.*, 2012).

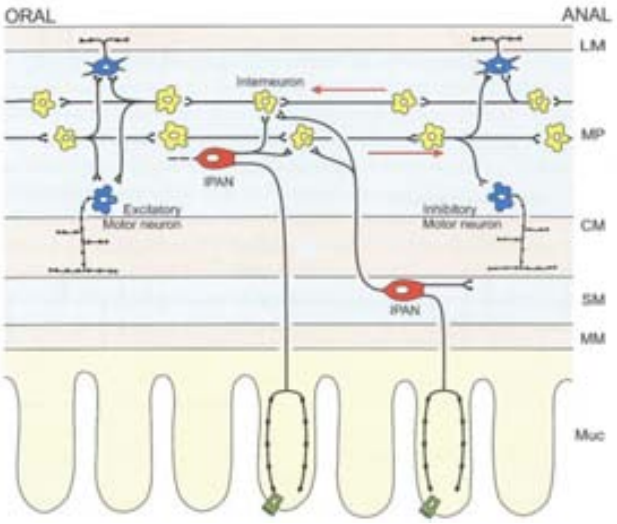

Figure 5. Neurones involved in the peristaltic reflex (simplified scheme). LM: longitudinal muscle; MP: myenteric plexus; CM: circular muscle; SM: submucosal plexus; MM: muscularis mucosae; Muc: mucosa. Red neurons: intrinsic primary afferent neurons (IPANs), yellow neurons (interneurons) and blue neurons (excitatory and inhibitory motor neurons). Adapted from Furness, 2006.

NEUROTRANSMITTERS IN THE GUT

ACh and tachykinins (TKs) are the main NTs released by excitatory motor neurones (Furness, 2000), whereas nitric oxide (NO), adenosine-5'-triphosphate (ATP) or a related purine, vasoactive intestinal polypeptide (VIP) and pituitary adenylyl cyclase activating peptide (PACAP) are the main NTs released by inhibitory motor neurones (Keef *et al.*, 1993;Grider *et al.*, 1994;Pluja *et al.*, 2000;Gallego *et al.*, 2008b). Hydrogen sulphide (H_2S) has been recently proposed as a putative NTs in the GI tract (Teague *et al.*, 2002;Schicho *et al.*, 2006;Tang *et al.*, 2013) and belongs to the family of gasotransmitters together with carbon monoxide (CO) and NO.

Excitatory transmitters trigger inward currents in SMCs via non-selective cation channels that provoke membrane potential depolarization (excitatory junction potential or EJP) and increase the probability of L-type calcium channels opening. On the contrary, inhibitory NTs cause outward currents through potassium channels elicit a smooth muscle hyperpolarization (inhibitory junction potential or IJP) and the open probability of calcium channels is reduced (Sanders, 2000). In "non-adrenergic, non cholinergic" (NANC) conditions, electrical field stimulation (EFS) applied to colonic strips elicits a transient IJP composed by two components: a fast and phasic component mediated by ATP or a related purine (fast inhibitory junction potential or IJPf) and a slow and more sustained component mediated by NO (slow inhibitory junction potential or IJPs) (Pluja *et al.*, 1999;Gallego *et al.*, 2006;Gallego *et al.*,

2008b;Burnstock, 2008). In "non-nitrergic, non-purinergic conditions" (i.e. in the presence of antagonists that block NO synthesis and the target receptor for the purinergic transmitter), the response observed when EFS is applied is an EJP (Gil *et al.*, 2013b).

It has been reported that SMCs can receive direct and indirect (through ICCs) neuronal input from motor neurones (Mitsui & Komuro, 2002). The recently proposed "intercalation" theory suggests that an ICC or a platelet derived growth factor receptor α-positive cell (PDGFRα+ cell, also known as fibroblast-like cell) is placed in-between motor neurons and SMC (Burns *et al.*, 1996;Kurahashi *et al.*, 2011;Peri *et al.*, 2013). PDGFRα+ cells have a similar distribution pattern to ICCs. They are excitable cells expressing small conductance calcium-activated potassium channels (sKca) and $P2Y_1$ receptors and therefore they have been proposed as mediators of purinergic inhibitory neurotransmission (Kurahashi *et al.*, 2011;Sanders *et al.*, 2012). Nevertheless, a consensus does not exist and some authors do not support the "intercalation" theory (Huizinga *et al.*, 2008;Goyal & Chaudhury, 2010;Zhang *et al.*, 2010a).

EXCITATORY NEUROTRANSMITTERS

It is established that excitatory motor neurons homogenously express choline acetyl transferase responsible for the synthesis of ACh but most of them also co-express TKs. Neurokinin A (NKA), substance P (SP) and NKB are the major members of the peptide family of mammalian TKs (Costa *et al.*, 2000;Lecci *et al.*, 2002;Holzer & Holzer-Petsche, 2001). ACh and TKs are the main excitatory NTs that elicit smooth muscle contraction acting postjunctionally on muscarinic M_2 and M_3 receptors and on tachykinergic NK_1 and NK_2 receptors respectively. In addition, a residual excitatory effect mediated by activation of $P2X_1$ receptor by ATP has also been described (Lecci *et al.*, 2002;Sanders *et al.*, 2012).

Acetylcholine

ACh is the most efficient intestinal excitatory NT in terms of intensity of stimulus needed to evoke contraction, latency to contraction and contraction amplitude (Maggi *et al.*, 1997;Lecci *et al.*, 2002). Cholinergic response was described in guinea-pig colon where, after blocking inhibitory neurotransmission by adding apamin, guantethidine, nitroarginine and nifedipine, a single pulse of EFS induced an EJP and the correspondent smooth muscle contraction, both being atropine-sensitive (Maggi *et al.*, 1997).

Five subtypes of muscarinic receptors have been described (M_1 to M_5), which may be co-acting postsynaptically in non-neuronal effector cells. GI smooth muscle express predominantly M_2 (70-80%) and M_3 (20-30%) (Lecci *et al.*, 2002), being M_3 the predominantly involved in GI smooth muscle contraction with a minor but concomitant participation of M_2 (Tobin *et al.*, 2009). M_3 is a GPCR that leads to calcium increase and, therefore, to contraction through PLC activation, IP_3 production and protein kinase C (PKC) activation (Lecci *et al.*, 2002;Tobin *et al.*, 2009). Accordingly, a significant reduction on carbachol-induced contractions has been reported both in bladder and intestine of M_3 receptor KO mice (Matsui *et al.*, 2000).

In addition to smooth muscle contraction, muscarinic receptors are involved in other physiologically relevant processes in the GI tract as secretion or blood flow. A role for muscarinic receptors on pathophysiological processes has also been reported: ACh may exert a dual pro-inflammatory (by inducing release of prostanoids and inflammatory substances) and an anti-inflammatory action (by inhibiting the production of tumour necrosis factor) (Tobin *et al.*, 2009).

Tachykinins

TKs act as NANC excitatory transmitters in mammalian respiratory, genitourinary and GI tract (Maggi, 2000). Three tachykinergic GPCR have been identified: NK_1, NK_2 and NK_3, which are mainly activated by SP, NKA and NKB respectively. The three receptors are internalized after agonist binding (Maggi, 2000;Lecci *et al.*, 2002; Vannucchi & Evangelista 2013). Although the distribution may differ depending on the species, region of the gut and cell type, the main localization of the three TK receptors in the GI tract is: NK_1 on neurons, ICC, SMC, glands and enterocytes, NK_2 on SMCs and NK_3 in neurons (Holzer & Holzer-Petsche, 1997;Furness & Sanger, 2002;Maggi, 2000; Vannucchi & Evangelista 2013). Human species shares a similar regional distribution of TKs with other mammals, having low concentrations of SP and NKA in esophagus, intermediate in stomach and high in the intestine (Holzer & Holzer-Petsche, 1997).

TK receptor activation can trigger non-selective cation channels opening and membrane depolarization that triggers VDCC activation and the correspondent influx of intracellular and extracellular calcium that evokes contraction (Sanders, 2000). In most intestinal segments, the atropine-resistant component of the contraction is due to activation of NK_1 or NK_2 receptors. The relative contribution of each receptor depends on the characteristics of the stimulus and the region of the gut. It has been described that brief trains

of EFS lead to activation of NK_1 whereas long-lasting trains elicit activation of both NK_1 and NK_2 receptors. It has also been reported that the tachykinergic response described in the guinea-pig small intestine is markedly different from the one observed in the colon, with involvement of both NK_1/NK_2 receptors in a different proportion in each gut segment (Maggi, 2000). On the contrary, the EFS-induced tachykinergic response in human sigmoid colon involves mainly NK_2 receptors, suggesting a major role for NKA (Cao *et al.*, 2000;Auli *et al.*, 2008).

The involvement of NK_2 receptors in pathophysiological processes has been studied in animal models of inflammation and human GI diseases. A reduction in the immunostaining in rat circular muscle and a decreased contractile response to TKs has been observed in inflammatory bowel syndrome (IBS) patients (Lecci *et al.*, 2002). Morevover, an over-expression of NK_1 and NK_2 receptors during GI inflammatory processes has been reported, and a role for NK_2 receptors in visceral hyperalgesia has been proposed (Vannucchi & Evangelista 2013).

ACh may be a predominant excitatory NT when compared to TKs. In the presence of blockers of inhibitory neurotransmission, single pulse of EFS is able to induce an atropine-sensitive EJP (Gil *et al.*, 2013b) and the correspondent cholinergic contraction (Maggi *et al.*, 1997). Nevertheless, trains of pulses are needed to induce a tachykinergic response, suggesting the need for higher intensity of stimulus to induce TK release (Maggi *et al.*, 1997). This phenomenon correlates to the higher effectiveness of antimuscarinic agents as constipative agents when compared to NK_1/NK_2 antagonists in the human and guinea-pig colon (Tonini *et al.*, 2001;Campos *et al.*, 2001;Lecci *et al.*, 2002).

INHIBITORY NANC NEUROTRANSMITTERS

Purinergic neurotransmitter

A compound could only be identified as a NT after a multidisciplinary approach that established four characteristics: 1- its presence and/or synthesis in neurons, 2- its release upon artificial or physiological stimulation, 3- the presence of specific receptors in effector cells (as smooth muscle or ICCs) and 4- the blockade of the physiological motor response by specific antagonists (Lecci *et al.*, 2002). Accordingly, it may be feasible that the exogenous addition of the compound could reproduce the effects of the endogenously released NT although it is important to bear in mind that activation of extrajunctional receptors may occur and thus the response elicited may not exactly mimic the endogenous transmitter (Jimenez *et al.*, 2014).

At the moment, the identity of the purinergic NT is still under debate (Hwang *et al.*, 2011;Goyal, 2011;Jimenez *et al.*, 2014). Adenosine 5'-triphosphate (ATP) molecule, established as an intracellular energy source involved in a huge variety of metabolic cycles, was proposed as a NANC NT in the GI tract by Burnstock in the 70s (Burnstock *et al.*, 1970;Burnstock, 1972). Nowadays, the term "ATP or a related purine" is commonly used in the literature due to the fact that ATP molecule is quickly metabolized by ecto-nucleotidases to adenosine 5'-diphosphate (ADP), adenosine monophosphate (AMP) and adenosine (Magalhaes-Cardoso *et al.*, 2003;Zimmermann, 2006;Duarte-Araujo *et al.*, 2009). Other purines as β-nicotinamide adenine dinucleotide (β-NAD) and/or its metabolic product adenosine diphosphate ribose (ADP-ribose) have also been recently proposed as purinergic NTs (Mutafova-Yambolieva *et al.*, 2007;Gustafsson *et al.*, 2011;Hwang *et al.*, 2011;Durnin *et al.*, 2012;Durnin *et al.*, 2013). Indeed, the four putative candidates (ATP, ADP, β-NAD and ADP-ribose) to act as NTs are released after EFS-evoked nerve stimulation of the gut (Mutafova-Yambolieva *et al.*, 2007;Hwang *et al.*, 2011;Mutafova-Yambolieva, 2012;Durnin *et al.*, 2013).

$P2Y_1$ receptors, which have been localized in neurons, SMCs and PDGRα+ cells in the gut (Monaghan *et al.*, 2006;Gallego *et al.*, 2006;Giaroni *et al.*, 2002;Kurahashi *et al.*, 2011), have demonstrated to play a crucial role in purinergic neuromuscular transmission in the GI tract of several species, including pig small intestine (Gallego *et al.*, 2008c), human colon (Gallego *et al.*, 2006;Gallego *et al.*, 2011) and rat colon (Grasa *et al.*, 2009). The relevance of $P2Y_1$ purine receptor as the mediator of purinergic GI smooth muscle relaxation has been demonstrated by means of a pharmacological approach and studies in $P2Y_1$ KO mice (Gallego *et al.*, 2006;Burnstock, 2008;Gallego *et al.*, 2011;Gallego *et al.*, 2012;Hwang *et al.*, 2012;Gil *et al.*, 2013a). The administration of $P2Y_1$ antagonists blocks the purinergic relaxation and the purinergic IJP in the following rank of potency MRS2179<MRS2279<MRS2500 (Boyer *et al.*, 2002;Kim *et al.*, 2003;Gallego *et al.*, 2011). Moreover, in $P2Y_1$ KO mice the purinergic component of the IJP is absent, and alterations of the gut motility are observed, giving more evidence to the critical role of $P2Y_1$ receptors in gut homeostasis (Hwang *et al.*, 2012;Gallego *et al.*, 2012;Gil *et al.*, 2013a). Taken together, these findings suggest that, although there is a general consensus about the critical role of the $P2Y_1$ receptor as target of the endogenous purinergic mediator and that the release of several purinergic substances by neurons is proven, the endogenous purine(s) that carries out the activation of $P2Y_1$ receptor has not still been clearly identified.

Purine receptors

Purine receptors are classified in two families: adenosine receptors (P1) and ATP/ADP/udidine 5'-diphosphate (UDP) and udidine 5'-triphosphate (UTP) receptors (P2). P2-purinoreceptors include two groups: P2X and P2Y. P2X receptors are ligand-gated ion channels and are classified in 7 subtypes ($P2X_1$-$P2X_7$), while P2Y are GPCR receptors and 8 subtypes are recognized ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) (Abbracchio *et al.*, 2006;Burnstock, 2008;Alexander *et al.*, 2011). Ionotropic P2X receptors are non-selective cation channels that, when activated, evoke a robust influx of Na^+ and Ca^{2+} inside the cell leading to depolarization and activation of intracellular calcium-dependent signalling cascades. Activation of metabotropic P2Y receptors leads to initiation of several intracellular signalling cascades, including activation of PLC and adenylyl cyclase (Zimmermann, 2006). $P2Y_1$ downstream pathway involves PLC activation, increase in IP_3 production and release of intracellular calcium from the sarcoplasmic reticulum (Burnstock, 2014a).

Ectonucleotidases may be involved in P2 receptor signalling as they are able to break down nucleotides responsible for activation of P2X and P2Y receptors, as ATP, ADP, UTP or UDP. Ecto-nucleoside triphosphate diposphohydrolase (NPTDase) family of ectonucleotidases, mainly NTPDase 1, 2, 3 and 8, placed in the surface of the plasma membrane, appear to be relevant to the control of P2 receptor signalling since its enzymatic activity hydrolyzes nucleotides at the range of concentration that activates P2 receptors. NTPDase1 hydrolyzes equally ATP and ADP, NTPDase2 is acting preferentially on triphosphonucleotides and NTPDase3 and 8 activity is intermediate between NTPDase1 and 2 (Kukulski *et al.*, 2005).

$P2X_1$, $P2X_2$, $P2X_4$, $P2X_7$, $P2Y_1$ and $P2Y_2$ receptors have been localized in smooth muscle (Burnstock, 2012). Some of the main P2X and P2Y receptor endogenous and synthetic agonists and antagonists are described in Table 2 and 3:

Table 2. Endogenous/synthetic agonists and antagonists of the P2X receptors.

Receptor	*Endogenous agonists*	*Synthetic agonists*	*Antagonists*
$P2X_1$	ATP	BzATP>2-MeSATP>α,β-meATP	NF449>NF023,NF279
$P2X_2$	ATP	ATPγS>2-MeSATP>α,β-meATP	iso-PPADS>PPADS>Suramin
$P2X_3$	ATP	2-MeSATP>α,β-meATP>BzATP	iso-PPADS>PPADS
$P2X_4$	ATP	α,β-meATP>2-MeSATP	PPADS
$P2X_5$	GTP	2-MeSATP=ATPγS>α,β-meATP	Suramin, PPADS
$P2X_7$	ATP	BzATP>2-MeSATP>α,β-meATP	MRS2427, O-ATP

>,greater potency; (=) or (,) equal potency; ATP, adenosine 5'-triphosphate; GTP, guanosine 5'-triphosphate; α,β-meATP, α,β-methylene adenosine 5´-triphosphate; 2-MeSATP, 2-methylthio-adenosine-5'-triphosphate; BzATP, 2'-3'-O-(4-benzoyl-benzoyl)- adenosine 5´-triphosphate; ATPγS, adenosine-5-(*γ*-thio)-triphosphate. Adapted from Burnstock, 2012;Burnstock, 2014b.

Table 3. Endogenous/synthetic agonists and antagonists of the P2Y receptors.

Receptor	*Endogenous agonists*	*Synthetic agonists*	*Antagonists*
$P2Y_1$	ATP>ADP	MRS2365>2-MeSADP> ADPβS>2-MeSATP	MRS2500>MRS2279>MRS2179
$P2Y_2$	UTP>ATP	2-Thio-UTP> UTPγS	AR-C126313>suramin>reactive blue2
$P2Y_4$	UTP>ATP	UTPγS	ATP>Reactive blue 2>suramin,PPADS
$P2Y_6$	UDP>UTP>ADP > ATP	3-phenacyl-UDP	MRS2578>reactive blue 2, PPADS
$P2Y_{11}$	ADP>ATP, β-NAD	ATPγS>ARC67085MX> BzATP>2-MeSATP	NF157>suramin>reactive blue 2
$P2Y_{12}$	ADP > ATP	2-MeSADP	Cangrelor>reactive blue 2
$P2Y_{13}$	ADP>ATP	2-MeSADP>2-MeSATP	Cangrelor >MRS2211
$P2Y_{14}$	UDP>UDP-glucose> UDP-galactose	MRS2690	

>,greater potency; (=) or (,), equal potency; ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; β-NAD, β-nicotinamide adenine dinucleotide; ADPβS, adenosine 5'-[β-thio]diphosphate trilitium salt; ATPγS, adenosine-5-(*γ*-thio)-triphosphate; UDP, uridine 5'-diphosphate; UTP, uridine-5'-triphosphate; 2-MeSATP, 2-methylthio-adenosine-5'-triphosphate; 2-MeSADP, 2-methylthio-adenosine 5'-diphosphate; BzATP, 2'-3'-O-(4-benzoyl-benzoyl)-adenosine 5´-triphosphate; Cangrelor, AR-C69931MX. Adapted from Abbracchio *et al.*, 2006;Burnstock, 2012;Burnstock, 2014b.

Purine receptor antagonists

Specific subtype antagonists are needed to characterize purine receptors involved in neuromuscular transmission in the gut. Suramin, PPADS and reactive blue have been commonly used as purinoreceptor antagonists; nevertheless, they do not discriminate between P2Y and P2X receptors. Other antagonists as NF023 or iso-PPADS exhibit higher

selectivity for P2X versus P2Y receptors (Ralevic & Burnstock, 1998). In 1998, Camaioni et al. synthesised MRS2179, a competitive and selective $P2Y_1$ antagonist (Boyer *et al.*, 1998;Camaioni *et al.*, 1998). This compound was used to prove that $P2Y_1$ receptor is responsible for the fast component of the IJP (IJPf) in the human colon (Gallego *et al.*, 2006;Gallego *et al.*, 2008b), pig small intestine (Gallego *et al.*, 2008c) and guinea-pig ileum (Wang *et al.*, 2007). However, MRS2179 may not exclusively antagonize $P2Y_1$ receptors, since in 2009 Pugliese et al. reported a blockade of GPR17 orphan receptor mediated responses (Pugliese *et al.*, 2009). In addition, high concentrations (up to 10 μM) of MRS2179 are needed to block the IJPf in murine internal anal sphincter (McDonnell *et al.*, 2008) and caecum (Zizzo *et al.*, 2007), as well as the non-nitrergic relaxation in the human small intestine (Undi *et al.*, 2009). Interestingly, new competitive and specific $P2Y_1$ antagonists, MRS2279 and MRS2500, have been synthesised. MRS2279 and MRS2500 show higher affinity for $P2Y_1$ receptor (with the lowest equilibrium dissociation constant for MRS2500 (K_B=1.74 nM in human) and do not exhibit non-selective effects on other P2Y receptors (Boyer *et al.*, 1998;Boyer *et al.*, 2002;Kim *et al.*, 2003;Cattaneo *et al.*, 2004;Camaioni *et al.*, 1998). MRS2279 and MRS2500 have been useful pharmacological tools to determine the relevant role of $P2Y_1$ receptors in the neuromuscular transmission in the gut (Gallego *et al.*, 2011).

In addition to the above mentioned, it should be taken into account that other P2Y receptor agonists and antagonists may be useful instruments to determine the putative implication of other purine receptors in endogenous inhibitory responses in the GI tract (see table 2 and 3).

Potassium channels and inhibitory neurotransmitters

Several types of K^+ channels are expressed in SMCs in the GI tract: delayed rectifier K^+ channels (Kv), large conductance calcium activated K^+ channels (BK) and sKca channels. K^+ channels are regulated by physiological stimuli, for instance sKca and BK are activated by intracellular calcium increase. Activation of postjunctional $P2Y_1$ receptor possibly activates sKca channels that in turn lead to hyperpolarization. Apamin, a sKca blocker, has been an important pharmacological tool to investigate inhibitory neurotransmission in the GI tract. Apamin causes smooth muscle depolarization and increases motility (Spencer *et al.*, 1998;Sanders, 2008;Sanders *et al.*, 2012;Gil *et al.*, 2012). In guinea-pig taenia caeci, it has been reported a depolarization of the RMP and a conversion of the IJP into an EJP after apamin administration (Zhang & Paterson, 2005). Apamin has been used as a tool to differentiate between the fast and the slow IJP, and the apamin-sensitive component has been associated

to the purinergic (fast) IJP (Zagorodnyuk & Maggi, 1994;Bennett, 1997;Pluja *et al.*, 1999;Serio *et al.*, 2003a). However, other studies support that apamin can also partially mediate nitrergic neuromuscular transmission (Keef *et al.*, 1993;Xue *et al.*, 1999;Gil *et al.*, 2012) and that the purinergic IJP is not completely sensitive to apamin in human and mouse colon (Gallego *et al.*, 2006;Zhang *et al.*, 2010b;Hwang *et al.*, 2011).

GASOTRANSMITTERS

In the 90s a new paradigm for cell-to-cell signal transduction was initiated with the discovery that the gaseous compound NO could be released specifically from nerves to transmit the signal after nerve stimulation. Soon after, CO was also recognized as the second gaseous NT with similar activity to NO. With the recent acknowledgement of H_2S as the third gaseous transmitter, the term "gasotransmitter" has been attributed to the gases acting as neurally released transmitters (Wang, 2002;Kasparek *et al.*, 2008). In 2002, Rui Wang presented the criteria to define gasotransmitters: 1- they are small molecules of gas (like NO or CO); 2- they are freely permeable to membrane and their effects will not rely on cognate membrane receptors; 3- they are endogenously and enzymatically generated in a regulated manner; 4- they have well-defined specific functions at physiologically relevant concentrations (for instance, both NO and CO participate in vasorelaxation and synaptic transmission in the CNS); 5- their cellular effects may or may not be mediated by second messengers but they should have specific cellular and molecular targets (for instance NO and CO activate BK channels in plasma membrane either directly or mediated by the cyclic guanosine monophosphate (cGMP) pathway) (Wang, 2002;Wu *et al.*, 2002).

Opposite to other signaling molecules, gasotransmitters are not stored in vesicles released by exocytosis: they are synthesized as needed in an extraordinarily regulated enzymatic process. To reach its target, instead of binding to plasma membrane receptors they diffuse into adjacent cells. But maybe the most unique feature of gasotransmitters relates to the molecular signalling mechanism to their targets. Classically, messenger molecules (for instance hormones and NTs) act through amplifying signalling cascades. By contrast, gasotransmitters elicit a chemical modification of intracellular proteins, therefore, they affect cellular metabolism in a more immediate fashion (Mustafa *et al.*, 2009). Accordingly, NO, CO and H_2S fulfil, at least partially, the criteria to be gasotransmitters: they are not stored within the cell in the classic presynaptic vesicles, permeate through cell membranes and activate signalling mechanisms independent of membrane receptors. The three of them are synthesized in the GI tract and have not only a physiologically relevant action but also a

potentially toxic activity at supra-physiologic concentrations (Kasparek *et al.*, 2008;Lowicka & Beltowski, 2007).

Nitric oxide

In 1980, Furchgott and Zawadzki proposed that activation of muscarinic receptors by exogenously added ACh was stimulating release of a substance(s) that caused relaxation of the vascular smooth muscle. This substance was named endothelium-derived relaxing factor (Furchgott & Zawadzki, 1980;Furchgott, 1983). In 1987, endothelium-derived relaxing factor was identified as NO (Ignarro *et al.*, 1987). Three years later, Bult et al. proposed NO to be the NANC inhibitory NT in the GI tract (Bult *et al.*, 1990).

NO is a free radical signaling molecule generated from L-arginine by a family of P450-like enzymes termed "NO synthases" (NOS). There are three independent genes encoding neuronal, endothelial and inducible NOS (nNOS, eNOS and iNOS respectively) (Shah *et al.*, 2004). The influence of NO on GI motility is largely controlled by nNOS, eNOS regulates NO actions on vascular function and iNOS-derived NO production is related to inflammatory or carcinogenic processes (Stuehr, 1999). Due to its lipophilic nature, NO diffuses quickly through plasma membranes, and its most characterized downstream signalling pathway relates to guanylyl cyclase (GC). NO binds to GC heme moiety stimulating the enzyme to produce cGMP second messenger, which in turn activates cGMP-dependent protein kinases, ion channels or phosphodiesterases (Denninger & Marletta, 1999;Friebe & Koesling, 2003;Dhaese *et al.*, 2008;Friebe & Koesling, 2009;De Man *et al.*, 2007;Groneberg *et al.*, 2011;Lies *et al.*, 2014). Although cGMP-independent mechanisms have also been attributed to NO (Keef *et al.*, 1993;Bolotina *et al.*, 1994;Wanstall *et al.*, 2005), their physiological role is still under debate (Friebe & Koesling, 2009). Indeed, it has been recently reported that GC KO mice totally lack NO-induced relaxation (Lies *et al.*, 2013a) and that remaining NO-induced effects in the presence of the GC blocker ODQ may not necessarily prove cGMP independence (Lies *et al.*, 2013b).

NO acts as an intracellular and intercellular messenger with important functions in numerous physiological and pathophysiological processes that occur in the GI tract, including motility, mucosal function, inflammatory responses, neoplasm and blood flow regulation. NO is also considered an important NANC NT in GI tract: NO produced by nNOS mediates the slow component of the IJP (IJPs) and causes smooth muscle relaxation in several areas of the gut and different species (Keef *et al.*, 1993;Xue *et al.*, 1999;Pluja *et al.*, 1999;Serio *et al.*, 2003b;Gallego *et al.*, 2008b;Lecea *et al.*, 2011;Opazo *et al.*, 2011). Data from human studies

indicate that NO inhibits esophageal smooth muscle function and mediates gastric accommodation reflex in humans (Tack *et al.*, 2002;Lecea *et al.*, 2011). $N^{(\omega)}$-nitro-L-arginine (L-NNA) (a NOS inhibitor) and ODQ (a GC blocker) inhibit IJPs, indicating that NO participates in the co-transmission process along with the purinergic agonist responsible for the IJPf (Goyal & He, 1998;Gallego *et al.*, 2008b).

Carbon monoxide

Two haem oxigenase enzymes, haem oxigenase-1 (HO-1) and haem oxigenase-2 (HO-2) are located in the endoplasmic reticulum and catalyse the synthesis of CO from Fe protoporphyrin IX. HO-1 is the inducible isoform and HO-2 is constitutively expressed (Maines, 1997). In physiological conditions, HO-1 is expressed at very low levels in the GI tract. However, HO-2 expression has been reported to be throughout the healthy gut in different species (Gibbons & Farrugia, 2004).

Like NO, CO activates GC but its efficacy and potency is lower than NO. CO binds to the haem at the active site of GC with a consequent increase in the cGMP levels (Gibbons and Farrugia 2004). However, as in the case of NO, cGMP-independent mechanisms of action have been described as well (Althaus *et al.*, 2009). CO causes hyperpolarization of SMC by increasing outward K^+ channels in the human and canine intestine (Farrugia *et al.*, 1993;Farrugia *et al.*, 1998). As CO elicits inhibitory actions, it has been proposed as an endogenous hyperpolarizing mediator in the GI tract (Szurszewski & Farrugia, 2004). CO is required in the membrane potential gradients along and across the GI muscle layers in the stomach, small intestine and colon. This voltage gradient regulates SMC contractility across the thickness of the gut wall and only allows the contraction of the more depolarised cells in response to a stimulus (Farrugia *et al.*, 2003;Gibbons & Farrugia, 2004).

Hydrogen sulphide

Most studies performed in the past have been devoted to the toxic effects of H_2S elicited by inhibition of the mitochondrial cytocrome c oxidase (Reiffenstein *et al.*, 1992). Recently, H_2S has been proposed as the third gasotransmitter with potential physiological roles (Lowicka & Beltowski, 2007). H_2S is endogenously produced and elicits physiologically relevant actions in several parts of the body, for instance: a) cardiovascular system, where H_2S is considered a second endothelium-derived hyperpolarizing factor (Tang *et al.*, 2013) that has proven to reduce blood pressure after intravenous administration (Zhao *et al.*, 2001) b) CNS, where H_2S is found in relatively high concentrations and facilitates long term potentiation in

the hippocampus (Abe & Kimura, 1996); c) urogenital system, where H_2S causes contraction in the rat urinary bladder through a neurogenic mechanism (Patacchini *et al.*, 2004); d) respiratory system, where H_2S may participate in a neurogenic inflammatory response mediated by transient receptor potential cation channel type V1 (TRPV1) channels in guinea-pig airways (Trevisani *et al.*, 2005); e) immune system, where H_2S may play an anti-inflammatory role since inhibition of its synthesis rapidly increases leukocyte adherence in rat venules (Zanardo *et al.*, 2006) and f) digestive system, where recent studies have suggested important roles for H_2S as mucosal defense, contribution to resolution of colitis or inhibition of motility (Gallego *et al.*, 2008a;Wallace *et al.*, 2009;Linden *et al.*, 2010;Gil *et al.*, 2013b). Nevertheless, H_2S physiological relevance is still under debate (Jimenez, 2010;Linden, 2014).

Although it is well known that H_2S can be synthesized by luminal bacteria, it is also endogenously produced in mammalian cells mainly by the enzymes cystathionine β- synthase (CBS) and cystathionine γ-lyase (CSE). Both are pyridoxal phosphate dependent enzymes that use L-cysteine as the substrate for H_2S synthesis. A third synthesis route involves 3-mercaptopyruvate sulfurtransferase (3MST) combined with cysteine aminotransferase (Stipanuk & Beck, 1982;Shibuya *et al.*, 2009a;Shibuya *et al.*, 2009b). CSE and CBS may function as cellular redox sensors that increase H_2S production in response to intracellular oxidant load and 3MST activates in response to physiologic acidification (Linden *et al.*, 2010).

H_2S endogenous production has been demonstrated in the rodent GI tract and both CBS and CSE have been localized along the gut (Martin *et al.*, 2010;Gil *et al.*, 2011). Both enzymes are expressed in the mouse colonic mucosa whereas only CSE is expressed in the external muscle layers of the colon, with the maximum level of immunoreactivity in enteric neurons (Linden *et al.*, 2008). In rat colon, CSE (but not CBS) is also found in enteric neurons (Gil *et al.*, 2011). Moreover, CSE and CBS are located in guinea pig and human submucous and myenteric neurons, and CSE is also expressed in ICCs (Schicho *et al.*, 2006).

Commonly used inhibitors of H_2S synthetic enzymes are: D,L-propargylglycine (PAG),an inhibitor of CSE, and aminooxyacetic acid (AOAA) and hydroxylamine (HA), which are both inhibitors of CBS (Szabo, 2007;Linden *et al.*, 2010). These compounds are able to inhibit H_2S synthesis in tissue homogenates but have poor tissue permeability and low selectivity that may lead to side effects (Hosoki *et al.*, 1997;Jimenez, 2010;Linden *et al.*, 2010). For instance, the CBS inhibitor HA causes a cGMP-dependent smooth muscle relaxation and hyperpolarization in the rat colon and, therefore, it might be considered a NO donor (Correia *et al.*, 2000;Gil *et al.*, 2011).

Under physiological conditions (aqueous solutions at pH 7.4) one third of H_2S remains undissociated and two thirds dissociate into H^+ and HS^- (hydrosulfite ion). At the moment, it remains unclear which of the two molecules is responsible for the effects of the compound (Lowicka & Beltowski, 2007;Linden *et al.*, 2010). Moreover, an important evaporation occurs when H_2S is added to the medium (Kimura, 2010). Sodium hydrogen sulphide (NaHS) is a commonly used H_2S donor that dissociates to Na^+ and HS^-; HS^- partially binds H^+ to form undissociated H_2S (Lowicka & Beltowski, 2007). Exogenously added NaHS elicits physiologically relevant actions in the GI tract, as inhibition of spontaneous motility recorded in vitro both in human and rodent GI tract (Gallego *et al.*, 2008a;Gil *et al.*, 2013b).

H_2S has several putative targets that may be tissue and species dependent. It has been reported that H_2S potentiates NMDA receptors during repetitive nerve stimulation (Abe & Kimura, 1996). H_2S might also activate apamin-sentitive (sKca) and glybenclamide-sensitive K^+ channels (ATP-sensitive potassium channels (K_{ATP})) (Distrutti *et al.*, 2006;Gallego *et al.*, 2008a), Cav3.2 T-type channels and chloride channels in pro-nociceptive neurons (Matsunami *et al.*, 2009) and TRPV1 channels (Schicho *et al.*, 2006;Krueger *et al.*, 2010) and transient receptor potential cation channel type A1 (TRPA1) receptors (Ogawa *et al.*, 2012), also associated to nociception. A relationship between H_2S and the cholinergic pathway has been suggested too. Recently, Gil *et al.* have demonstrated that H_2S blocks the cholinergic EJP and the depolarization evoked by carbachol in rat colon (Gil *et al.*, 2013b) (Figure 6). In respiratory system, H_2S reversibly inhibits ACh-induced calcium oscillations responsible for the contraction of airway SMCs (Castro-Piedras & Perez-Zoghbi, 2013). Similarly, H_2S inhibits cholinergic-mediated contractions in guinea-pig ileum (Teague *et al.*, 2002). In addition to the previously commented, H_2S is also able to bind with high affinity to heme group in a similar way to NO, although H_2S might not activate soluble GC (Abe & Kimura, 1996). It has been postulated that H_2S may elicit its actions by S-sulfhydration of cysteins in target proteins, an analogous mechanism to S-nitrosilation induced by NO. However, whereas S-nitrosilation reduces enzymatic acivity, S-sulfhydration may evoke the opposite effect (Mustafa *et al.*, 2009).

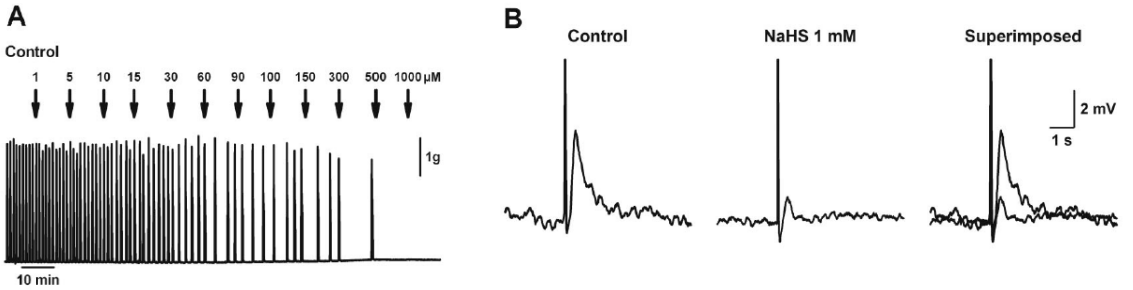

Figure 6. (A) Muscle bath recording showing the inhibitory effect of cumulative concentration-response curve of NaHS (in the presence of TTX 1 µM) on spontaneous contractions in circularly-oriented rat colonic strips devoid of mucosa and submucosa. (B) Intracellular microelectrode recording showing the EFS-evoked EJP in the presence of L-NNA 1mM and MRS2500 1 µM (Control) and the inhibitory effect of NaHS 1mM both in duration and amplitude of the EJP in rat colonic smooth muscle. Adapted from Gil *et al.*, 2013b.

H_2S is present in the lumen of the human large intestine at high concentrations (range of millimolar) due to the metabolism of luminal bacteria. However, due to the capability of fecal components to bind the sulphide, the amount of free H_2S molecules is relatively low (micromolar range). The colonic epithelial cells of the mucosa are not only a physical barrier protecting the organism from potentially harmful substances in the lumen of the GI tract but also a metabolic barrier that represents an excellent system for H_2S degradation. Colonic epithelium quickly oxidizes H_2S to thiosulfate, protecting from the harmful consequences of a toxic level of H_2S (Wallace *et al.*, 2012). The enzymatic activity involved in this metabolic process consists of sulphide quinone oxidoreductase followed by the action of sulfur dioxygenase and sulphur transferase (Blachier *et al.*, 2010). Due to the efficiency of colonic epithelium metabolism and under physiological conditions, the amount of H_2S that reaches the mucosa/submucosa is very low. However, when the epithelial barrier is dysfunctional or damaged, higher concentrations of H_2S trespass the mucosa and considerable effects on secretion, pain sensation, blood flow or even smooth muscle contractility may be observed (Wang, 2002;Schicho *et al.*, 2006;Wallace *et al.*, 2012).

PHARMACOLOGICAL TOOLS: MOTILITY DISORDERS

Functional bowel disorders are functional GI disorders with symptoms attributable to the middle or lower GI tract, including IBS, bloating, constipation and diarrhea (Longstreth *et al.*, 2006). IBS has a very high prevalence (affects approximately 10-15 % or more of the population worldwide) (Boeckxstaens *et al.*, 2014) and elicits a significant impairment of patients' quality of life (Lacy *et al.*, 2009). IBS has been defined as a chronic disorder characterized by abdominal pain, discomfort and/or bloating associated with disorders in

defecation (constipation, diarrhoea or mixed/alternated) (Longstreth *et al.*, 2006;Boeckxstaens *et al.*, 2014).

Due to the complexity of IBS and the poor understanding of its pathophysiology, the therapy is often a symptom-based approach (Drossman *et al.*, 2002). IBS pathophysiology is variable depending on the patient and may be associated to intestinal infection, inflammation, intestinal microflora, stress, diet and genetic predisposition. Approximately 25-75% of IBS patients have motility disorders and these transit abnormalities lead to low sensory thresholds. Among IBS patients exhibiting diarrhoea and abdominal pain, the number and amplitude contractions of high-amplitude is significantly increased compared to control, and this feature is more likely associated to pain (Drossman *et al.*, 2002). At the moment, the therapeutic targets identified in GI smooth muscle and the efficacious compounds are scarce (Sanders *et al.*, 2012). To treat abdominal pain and discomfort related to hypermotility, smooth muscle relaxants (also called antispasmodics or spasmolytic drugs) as otilonium bromide (OB) or anticholinergic drugs such as hyoscine are commonly used. Both OB and hyoscine have shown a significant effectiveness compared to placebo in clinical trials and the need of smooth muscle relaxants in management of IBS has been demonstrated (Poynard *et al.*, 2001;Ford *et al.*, 2008;Clave *et al.*, 2011).

Antispasmodic agents: Otilonium Bromide

Antispasmodic agents are a heterogeneous group of drugs that decrease the tone and contractility of intestinal smooth muscle. Depending on their main mechanism of action, antispasmodic agents are usually divided into three groups: direct smooth muscle relaxants or antispasmodics (also named musculotropic), calcium channel antagonists and anticholinergic/antimuscarinic agents (Tack *et al.*, 2006;Boeckxstaens *et al.*, 2014).

OB is a spasmolytic compound commonly used to treat the motor disorders present in gut diseases such as inflammatory bowel disease (IBD) (Forte *et al.*, 2012). Three mechanisms of action have been associated with OB effects: reduction of calcium entry into SMC that reduces smooth muscle contractility and dismotility, inhibition of calcium release from sarcoplasmic reticulum and reduction of visceral hypersensitivity due to interaction with tachykinergic (NK_2) receptors located in afferent nerves (Boeckxstaens *et al.*, 2014). It has been reported that OB reduces abdominal pain and discomfort (Battaglia *et al.*, 1998) and enhances sensory thresholds to recto-simoidal distension (Czimmer *et al.*, 2001). Other randomized clinical trials have also concluded that OB is a safe and efficacious drug that reduces the

frequency of abdominal pain and severity of abdominal bloating in IBS patients, with protection of symptom relapse even after discontinuation of the treatment (Chang *et al.*, 2011;Clave *et al.*, 2011).

Some musculotropic spasmolytics such as OB have the advantage of being usually inactive outside of the GI tract, opposite to other antispasmodic drugs such as anticholinergic agents (Forte *et al.*, 2012). Due to its quaternary ammonium structure, OB has poor systemic absorption after oral administration and therefore is almost completely (97.8%) eliminated in faeces (Evangelista, 2004;Boeckxstaens *et al.*, 2013). Indeed, after oral administration of radioactively marked OB, very low plasma levels but an effective penetration into the large intestine wall have been reported (Evangelista *et al.*, 2000). Moreover, after incubation of frozen sections of the GI tract (also with radioactively marked OB), the drug accumulated within the colonic and rectal smooth muscle but not in small intestine or stomach (Amenta *et al.*, 1991). Accordingly, OB has mainly a local effect, is devoid of serious side effects and has demonstrated effectiveness and tolerability when compared to other spasmolytic agents (Forte *et al.*, 2012).

The reported pharmacological properties of OB include L-type and T-type calcium channel blocker and anti-muscarinic and anti-tachykinergic activity (Santicioli *et al.*, 1999;Evangelista *et al.*, 1998;Martin *et al.*, 2004;Strege *et al.*, 2010;Strege *et al.*, 2004;Gallego *et al.*, 2010). Recently, a possible interaction of OB with SMC sodium channels has also been described (Strege *et al.*, 2010). Since L-type calcium channels have multiple involvements in SMC contractility, they are a strategic target for spasmolytic therapy. A competitive binding of OB to L-type calcium channels with micromolar affinity has been reported in rat colon (Evangelista *et al.*, 1998). Posterior patch-clamp studies have also demonstrated that OB blocks L-type channels in human jejunal and rat colonic SMCs (Strege *et al.*, 2004;Martin *et al.*, 2004). In the human colon, OB inhibited non-neural spontaneous RPCs, stretch-induced tone, EFS-induced off-contractions and KCl-induced calcium transients in isolated cells mainly by blocking L-type calcium channels (Gallego *et al.*, 2010). OB inhibits not only L-type but also T-type calcium channels: it has been reported a reversible inhibition of Cav 3.1, Cav 3.2 and Cav 3.3 channels expressed in HEK293 cells (Strege *et al.*, 2010) with a lower EC_{50} than the one required for L-type blockade (Strege *et al.*, 2004).

In addition to the inhibitory effect on calcium channels, OB has demonstrated antimuscarinic and antitachykinergic properties. It has been reported that OB binds with sub-micromolar affinity to muscarinic receptors (M_1, M_2, M_4 and M_5) in a radioligand binding assay and competitively to M_2 receptors in the rat colon (Evangelista *et al.*, 1998). Another study

demonstrated that OB inhibits ACh-induced calcium signals in isolated human colonic crypts and in Chinese hamster ovary cells stably expressing the cloned M_3 receptor (Lindqvist *et al.*, 2002). Moreover, in guinea-pig colon, OB concentration-dependently inhibited the contraction and depolarization elicited by a muscarinic agonist and also by NK_1 and NK_2 receptor agonists. This inhibition of NK_1 mediated responses by OB may not be associated to a direct effect on the receptor but to the blockade of a downstream process, such as L-Type calcium channels activation, due to the fact that OB had no effect in the presence of nifedipine. However, in the case of NK_2 receptors, OB inhibitory effect might be the result of a competitive direct binding to the receptor (Santicioli *et al.*, 1999). It has been also reported that OB inhibits in a concentration-dependent manner the internalization of NK_2 receptor elicited by the presence of agonist in a similar way to the selective antagonist idobutant in the human colon (Cipriani *et al.*, 2011). Moreover, chronic treatment with OB can significantly affect the NK_1 receptor - SP - NO circuit, eliciting a progressive decrease in SP expression, a redistribution of the L-type calcium channels and NK_1 receptor and a compensatory increase of NOS expression in order to guarantee an adequate NO production (Traini *et al.*, 2013). In addition to the previously commented, a direct inhibitory effect of OB on nicotinic receptors present in bovine chromaffin cells has been described (Gandia *et al.*, 1996). However, in human sigmoid colon in vitro studies, an anti-nicotinic effect has not been observed since the inhibitory effect of OB on contractility was not modified by hexametonium (Gallego *et al.*, 2010).

PROSTAGLANDINS

Prostaglandins (PGs) and tromboxanes (TXAs) are prostanoids synthesized from the C20-unsaturated fatty acids such as arachidonic acid by two isoforms of the enzyme cyclooxygenase (COX): COX-1 and COX-2. After a physiological or pathophysiological stimulus, PGs and TXAs are synthesized and immediately released outside the cell membrane (leaving a little or any of the product inside the cell) to act in the vicinity of their production site to maintain local homeostasis. The COX reaction produces unstable endoperoxide intermediates (PGH_2 and PGG_2) that are metabolized by cell-specific isomerases and synthases to PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2. Some prostanoids, as PGI_2 and TXA_2, are spontaneously degraded to inactive compounds but others need enzymatic activity to be inactivated (Narumiya & FitzGerald, 2001;Sugimoto & Narumiya, 2007;Dey *et al.*, 2006). Prostanoids are lipidic compounds but they do not freely permeate the plasmatic membrane; a PG transporter, member of the organic anion transporter polypeptide family, has been identified and localized in a narrow range of cell types and it is postulated that other mechanisms of prostanoid

transport may exist. It is believed that prostanoids may exert their actions through membrane receptors in the cell surface or target cells, but actions on nuclear receptors have been also reported (Narumiya & FitzGerald, 2001).

The two COX isoforms are expressed in the GI tract in smooth muscle, lamina propria mononuclear cells, ICCs and myenteric neurons (Zimmermann *et al.*, 1998;Fornai *et al.*, 2005;Cosme *et al.*, 2000;Roberts *et al.*, 2001;Porcher *et al.*, 2004). Normal gut expresses high levels of the protective isoform COX-1 and low levels of COX-2 (Fornai *et al.*, 2005;Wang & DuBois, 2008) and in the presence of neoplasm, ulcer or inflammation, the expression of COX-2 is up-regulated. While COX-2 has been considered for many years an inducible enzyme (Habib *et al.*, 1993), a constitutive expression has been demonstrated in mouse, rabbit and human GI tract (Zimmermann *et al.*, 1998;Porcher *et al.*, 2004;Bernardini *et al.*, 2006). Recent studies have suggested that the constitutively expressed COX-2 relevantly contributes to the synthesis of PGs responsible for the regulation of smooth muscle contractility in murine proximal colon (Porcher *et al.*, 2004), although both isoforms might be able to act at neuronal level to modulate the contractile activity elicited by excitatory cholinergic pathways (Fornai *et al.*, 2006). It is possible that the concomitant oxidative stress found during inflammatory processes impairs COX-1 activity, and thus COX-2 may be playing a predominant role in the inhibitory control of the neuromuscular function of the colon during IBD (Roberts *et al.*, 2001;Fornai *et al.*, 2006).

PGs are synthesized throughout the human GI tract, and the following gradient of sensitivity to prostanoids has been described: stomach>distal ileum>sigmoid colon (Bennett *et al.*, 1981). Eight types and subtypes of membrane prostanoid receptors are present in mammals: the PGD receptor (DP), four subtypes of PGE receptor (EP_1, EP_2, EP_3, EP_4), the PGF receptor (FP), the PGI receptor (IP) and the TXA receptor (TP). All of them are GPCR with seven transmembrane domains. Different genes are encoding each receptor subtype and in the case of EP_3, FP and TP receptors, several splice variants exist differing in the C-terminal tails. These membrane prostanoid receptors can be classified depending on their neuromuscular effects. IP, DP, EP_2 and EP_4 lead to cyclic adenosine monophosphate (cAMP) increase and are considered “relaxant” receptors; TP, FP and EP_1 induce calcium mobilization by activation of phospholipase C and IP_3 production and are termed “contractile” receptors; and finally the EP_3 receptor evokes a reduction of cAMP levels and contraction and is termed the “inhibitory” receptor. Despite this classification, the effects triggered by activation of each receptor may differ depending on the concentration or structure of the ligand (Narumiya & FitzGerald, 2001;Dey *et al.*, 2006;Sugimoto & Narumiya, 2007).

The E type PG, particularly PGE_2, is the most common in different animal species, the most widely produced in the body and also the most functionally relevant (Sugimoto & Narumiya, 2007). PGE_2 regulates many physiological functions in the gut as mucosal protection, GI secretion and even motility. Nevertheless, PGE_2 has been also related to pathophysiological processes such as IBD and colorectal neoplasm (Takafuji *et al.*, 2000;Dey *et al.*, 2006;Wang & DuBois, 2008). Indeed, PGE_2 and other prostanoids might exert both pro-inflammatory and anti-inflammatory responses (Sugimoto & Narumiya, 2007).

Opposite to other prostanoids that have one single receptor, PGE_2 targets four different EP receptors (EP_1, EP_2, EP_3 and EP_4) (Narumiya & FitzGerald, 2001). PGE_2 binds potently to the four EP receptor subtypes, with Kd in the range of 1-40 nM. Conventionally used PGE_2 analogues are not specific for an EP receptor, although highly selective compounds have been developed by using cultured cell lines stably expressing each EP receptor subtype. An exception is butaprost, which binds specifically to EP_2 receptors (Sugimoto & Narumiya, 2007). The signalling pathway triggered after PGE_2 binding is different for every EP receptor subtype. EP_1 preferentially couples to Gq and leads to an increase of intracellular calcium levels. Differently, EP_2 and EP_4 signal through Gs, eliciting an increase of cAMP production. EP_3 main signalling pathway relies on Gi coupling and produces a reduction of cAMP (Dey *et al.*, 2006;Sugimoto & Narumiya, 2007;Fairbrother *et al.*, 2011). EP receptor expression levels in the body vary depending on the tissue and even on the cell type within the same organ. For instance, the study of tissue distribution of EP receptors in mouse revealed that EP_3 and EP_4 are the most widely distributed whereas EP_1 is restricted to several organs as kidney, lung and stomach and EP_2 is the least abundant (Sugimoto & Narumiya, 2007). Depending to their effects on the smooth muscle, the four EP receptors can be classified as relaxant EP_2 and EP_4 receptors and contractile EP_1 and EP_3 receptors (Narumiya *et al.*, 1999). The opposite downstream actions after activating one or another EP receptor and the different EP receptor expression in different tissues may explain the reported dual effects of PGE_2 on smooth muscle in different regions of the body, including the gut, and in different species (see Table 4):

Table 4. PGE_2 effects on smooth muscle in different tissues and species and EP receptor(s) involved.

PGE_2 effects	Tissue	Species	Receptor	References
relaxation	cerebral arteries	human	EP_4	Davis *et al.*, 2004
relaxation	trachea	mouse	EP_2	Fortner *et al.*, 2001
Contraction/relaxation (tone maintenance)	trachea	guinea-pig	EP_1/EP_2	Safholm *et al.*, 2013
Contraction/relaxation	Mesenteric/tail arteries	rat	EP_3 /EP_4	Kida *et al.*, 2013
Relaxation/contraction	Circular/longitudinal ileal muscle	human, guinea-pig and rat		Bennett *et al.*, 1968
Relaxation/contraction	Circular/longitudinal stomachic, ileal and colonic muscle	human		Bennett *et al.*, 1981
Contraction	Longitudinal colonic [(1,2)] and ileal [(2)] muscle	Mouse and human	EP_1 [(1)]; EP_1 and EP_3 [(2)]	Fairbrother *et al.*, 2011
Slow wave frequency increase	Stomach	mouse	EP_3	Forrest *et al.*, 2009
Generation of contractions	Small intestine	rabbit	EP_1 and EP_3	Grasa *et al.*, 2006
Generation of contractions	Longitudinal colonic muscle	rat	EP_1 and EP_3	Grasa *et al.*, 2006
Relaxation/contraction	Circular colonic muscle	dog	EP_3 /EP_4	Botella *et al.*, 1995

[(1)] human, [(2)] mouse

Apart from their known physiological relevance, PGs may play a role in several GI diseases such as IBD or colorectal cancer (Wang & DuBois, 2008). In IBS patients, a higher plasma level of arachidonic acid compared to controls has been reported (Clarke *et al.*, 2010). Moreover, EP receptor expression may be altered in some gut diseases, suggesting a role for PGE_2 triggered pathways in GI inflammation (Narumiya & FitzGerald, 2001;Sugimoto & Narumiya, 2007;Wang & DuBois, 2008). Indeed, an up-regulated expression of EP_4, EP_2, EP_3 receptors and EP_2, EP_1 receptors has been associated to colonic inflammation and neoplasm respectively (Takafuji *et al.*, 2000;Dey *et al.*, 2006). It has also been reported that the blockade of either PGE_2 synthesis or EP receptors impairs acute and chronic inflammation (Wang & DuBois, 2008;Sugimoto & Narumiya, 2007). In motility disorders such as slow-transit constipation, increased PGE_2 levels and up-regulated expression of EP receptors and COX-2 have been observed (Cong *et al.*, 2007). In addition, EP_2 and EP_4 receptor antagonists as well as COX-2 inhibitors have improved considerably the motility dysfunction associated to colonic obstruction (Lin *et al.*, 2012).

OBJECTIVES

In order to treat colonic motility disorders it is important to study mechanisms of smooth muscle relaxation that can be the basis of spasmolytic drugs. In the present work we characterized: 1- nerve mediated relaxation of the gut, 2-possible endogenous substances that have the ability to relax smooth muscle and 3- the mechanism of action of spamolytic drugs in use for the treatment of colonic disorders. In particular, we studied the responses and mechanism of action of purine receptor agonists, Hydrogen sulphide (H_2S), Prostaglandin E_2 (PGE_2) and Otilonium Bromide (OB) in the colon.

The principal objectives of this thesis were the following:

1. Pharmacological characterization of the endogenous purinergic transmitter acting on the $P2Y_1$ receptor and comparison with several purinergic agonists in human and rat colon.
2. Pharmacological characterization of PGE_2 inhibitory effects in the mouse colon.
3. Investigation of the mechanisms of action linked to the inhibitory effects of H_2S in the human colon.
4. Study of the mechanisms of action responsible for the spasmolytic activity of OB in the human and rat colon.

In order to accomplish these objectives, the main experimental procedures used in the present work have been organ bath technique to study the mechanical activity and microelectrode technique to study the smooth muscle membrane potential. Both excitatory and inhibitory neurotransmission can be characterized with these two techniques. Moreover, calcium imaging technique has also been used in order to study intracellular calcium responses evoked by drug administration to isolated human colonic smooth muscle cells (HCSMCs). These three in vitro techniques have been useful tools to investigate the neuromuscular interaction and to characterize these inhibitory pathways.

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CHAPTER 1

α,β-meATP mimics the effects of the purinergic neurotransmitter in the human and rat colon

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ABSTRACT

The purine receptor involved in inhibitory responses in the gastrointestinal tract has been recently identified. $P2Y_1$ receptor activation mediates the fast component of the inhibitory junction potential (IJPf) and the non-nitrergic relaxation. The aim of the present work has been to investigate which purinergic agonist better mimics endogenous responses. We used different agonist and antagonist of P2 receptors. Contractility and microelectrode experiments were used to compare the effects of exogenously added purines and electrical field stimulation (EFS)-induced nerve mediated effects in rat and human colonic strips. In rat colon, the IJPf and EFS-induced inhibition of contractions were concentration-dependently inhibited by the $P2Y_1$ antagonist MRS2500 but not by iso-PPADS or NF023 (P2X antagonists) up to 1 μM. In samples from human colon, EFS-induced inhibition of contractions was inhibited by either MRS2500 or apamin (1 μM) but not by iso-PPADS. In both species, α,β-meATP, a stable analogue of ATP, caused inhibition of spontaneous contractions. α,β-meATP effect was concentration-dependent (EC_{50}: 2.7 μM rat, 4.4 μM human) and was antagonised by either MRS2500 or apamin but unaffected by P2X antagonists. ATP, ADP, β-NAD and ADP-ribose inhibited spontaneous contractions but did not show the same sensitivity profile to purine receptor antagonists as EFS-induced inhibition of contractions. The effect of α,β-meATP is due to $P2Y_1$ receptor activation leading the opening of small conductance calcium-activated potassium channels. Accordingly, α,β-meATP mimics the endogenous purinergic mediator. In contrast, exogenously added putative neurotransmitters do not exactly mimic the endogenous mediator. Quick degradation by ecto-nuclease or different distribution of receptors (junctionally vs extrajunctionally) might explain these results.

INTRODUCTION

The purinergic neurotransmitter(s) (NTs) responsible for non-adrenergic, non-cholinergic (NANC) inhibitory responses in the gastrointestinal (GI) tract has still not been identified. Several purines including adenosine 5´-triphosphate (ATP), adenosine 5'-diphosphate sodium salt (ADP), β-nicotinamide adenine dinucleotide hydrate (β-NAD) or even adenosine 5'-diphosphoribose sodium salt (ADP-ribose) are nowadays possible candidates for this role (Burnstock *et al.,*1970; Mutafova-Yambolieva *et al.,*2007;Durnin *et al.,*2012). Recently, the receptor responsible for purinergic neuromuscular transmission has been identified. $P2Y_1$ receptor antagonists such as MRS2179, MRS2279 and MRS2500 have been valuable pharmacological tools to demonstrate the crucial role of $P2Y_1$ receptors in purinergic neuromuscular transmission (Gallego *et al.,*2006;Wang *et al.,*2007;Gallego *et al.,*2008a;Gallego *et al.,*2008b;Grasa *et al.,*2009). MRS2500 is considered the most potent $P2Y_1$ antagonist (Kim *et al.,* 2003;Cattaneo *et al.,*2004) and has proven to be inactive on other purine receptors such as P2X (Bradley *et al.,*2011;Doyle *et al.,*2014), $P2Y_{12}$ (Hechler *et al.,*2005) and $P2Y_{13}$ (Gao *et al.,*2010). Studies in knocked-out mice have confirmed $P2Y_1$ receptor relevance on purinergic junction potentials and smooth muscle relaxation in the colon (Gallego *et al.,*2012;Hwang *et al.,*2012), stomach and caecum (Gil *et al.,*2013). A consensus about the involvement of the $P2Y_1$ receptor in smooth muscle relaxation in the whole GI tract now exists (King, 2012;Gil *et al.,*2013;Goyal *et al.,*2013). The identification of $P2Y_1$ receptors has been crucial in understanding the process of co-transmission between purines and nitric oxide (NO) and in establishing pharmacological criteria for identifying potential agonists able to mimic endogenous responses (Gallego *et al.,*2006;Mutafova-Yambolieva *et al.,*2007;Gallego *et al.,*2011;Durnin *et al.,*2012;Gil *et al.,*2013).

α,β-methylene adenosine 5´-triphosphate lithium salt (α,β-meATP) is an unselective P2X receptor agonist (Alexander *et al.,*1999). The effect of α,β-meATP on smooth muscle excitability differs depending on the species and area of the GI tract. Consistent with an effect on P2X receptors, α,β-meATP produces smooth muscle depolarization and contraction. An excitatory junction potential due to activation of $P2X_1$ receptors has been reported in the guinea-pig taenia caeci (Zhang & Paterson,2005). α,β-meATP induces non-selective cation inward currents triggering smooth muscle depolarization and contraction in the canine colon (Lee *et al.,*2005). However, in other tissues, α,β-meATP causes smooth muscle hyperpolarization and/or relaxation (Zagorodnyuk *et al.,*1996;Storr *et al.,*2000;Ishiguchi *et al.,*2000;Giaroni *et al.,*2002;De Man *et al.,*2003;Van Crombruggen *et al.,*2007;King & Townsend-Nicholson,2008). The receptor involved in α,β-meATP effect is not clear and high

concentrations of P2X antagonists such as PPADS are often used for its identification. It is important to investigate if high concentrations of P2X antagonists can inhibit $P2Y_1$-mediated responses. Little is known about α,β-meATP effect in human smooth muscle excitability. In the jejunum, α,β-meATP causes hyperpolarization and partial inhibitory junction potential (IJP) desensitization (Xue *et al.,*1999) and in the human colon, the relaxation induced by α,β-meATP is blocked by high concentrations of MRS2179 (Auli *et al.,*2008), suggesting a possible involvement of $P2Y_1$ receptors.

Accordingly, the aim of this paper is to establish a pharmacological methodology to characterize the receptor involved in endogenous purinergic responses and to investigate which of the exogenously added purines better mimics the NT. This can be used in future studies where $P2Y_1$ agonists might be useful to treat purinergic motor disorders (Strong *et al.,*2010;Roberts *et al.,*2012).

MATERIALS AND METHODS

Rat tissue preparation

Male Sprague-Dawley rats (300-350 g; 8-10 weeks old) were kept at a constant room temperature (19-21 ºC) and humidity (60 %), with a lighting cycle of 12 h light/12 h dark and *ad libitum* access to water and food. Animals were stunned by a sharp blow to the head before being decapitated and bled. The mid colon was quickly placed in carbogenated Krebs solution. The mesenteric fat was removed and the colon was opened along the mesenteric border and pinned to a Sylgard base with the mucosa facing upwards. The mid colon was distinguished according to the longitudinal orientation of the folds of the mucosa (total length about 5 cm in the centre of the colon) taking into account the anatomical criteria previously described (Alberti *et al.,* 2005). Mucosa and submucosa layers were carefully removed and circular muscle strips were cut into strips 1 cm long and 0.3 cm wide. This procedure was approved by the Ethics Committee of the Universitat Autònoma de Barcelona.

Human tissue preparation

Tissue specimens of human sigmoid colon (n= 64) were obtained from patients (34 female and 30 male, average age 68) during colon resections of neoplasm. The person that performed the experiments and analyzed the tracings was initially not aware of this information and accordingly, gender and age were not considered as variables of this study. Colon segments from macroscopically-normal marginal 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 mucosa layer was gently removed. Circular muscle strips (10x4 mm) were cut. The patients provided informed consent. Ethics committee of the Hospital of Mataró (Barcelona, Spain) approved the experimental procedure.

Intracellular microelectrode recording

Muscle strips were pinned to the base of a Sylgard coated chamber, circular muscle side up, and continuously perfused with Krebs solution. Strips were allowed to equilibrate for approximately 1 h before recording. Circular smooth muscle cells (SMCs) were impaled with sharp glass microelectrodes filled with 3 M KCl (30-60 MΩ). Membrane potential was measured using standard electrometer Duo773 (WPI Inc., Sarasota, FL, USA). Tracings were displayed on an oscilloscope 4026 (Racal-Dana Ltd., Windsor, UK) and simultaneously digitalized (100 Hz) using PowerLab 4/30 system and Chart 5 software for Windows (all from

ADInstruments, Castle Hill, NSW, Australia). Nifedipine (1 μM) was used to abolish the mechanical activity and obtain stable impalements. Electrical field stimulation (EFS) was applied using two silver chloride plates placed perpendicular to the longitudinal axis of the preparation and 1.5 cm apart. Tissue incubated with L-NNA was used to elicit supramaximal IJPf using single pulses (pulse duration 0.3 ms, 32 V). α,β-meATP was added by superfusion. The amplitude of EFS-induced IJPf and α,β-meATP hyperpolarization was measured in the absence (control conditions) and in the presence of purine receptor antagonists (incubated during 15 min).

Mechanical studies

Spontaneous mechanical activity was studied in a 10 mL organ bath. Circularly-oriented 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. Mechanical activity was recorded by means of the transducer which was connected to a personal computer through an amplifier. Data were digitalized (25 Hz) using Data 2001 software (Panlab, Barcelona, Spain) coupled to an ISC-16 A/D card. A tension of 1 g (rat) or 4 g (human) was applied and the tissue was allowed to equilibrate for 1 h. After this period, strips displayed spontaneous phasic activity. In order to study purinergic responses, tissue was incubated with NANC Krebs containing L-NNA 1 mM. EFS was applied through two platinum electrodes placed on the support holding the tissue and had a total duration of 2 min (pulse duration 0.3 ms, frequency 5 Hz, amplitude 30 V in rat; pulse duration 0.4 ms, frequency 2 Hz, amplitude 50 V in human). The effect of several purine receptor antagonists on the EFS-induced inhibition of contractions was studied in different strips. The response to purine receptor agonists (α,β-meATP, ATP, ADP, ADP-ribose, β-NAD and ADPβS) was studied performing a cumulative concentration-response curve. The effect of α,β-meATP was also studied with a protocol using a single concentration of 10 μM. UTP and UDP-glucose were tested at 100 μM. The effect of purine agonists was studied both in control conditions and after incubation (15 min) with several purinergic antagonists, using different strips for each protocol. To estimate mechanical activity responses to drugs or EFS, the area under curve (AUC) of contractions from the baseline was measured before and after drug addition and before and during EFS. AUC is expressed as grams per min ($g \cdot min^{-1}$).

Solutions and drugs

The composition of the Krebs solution was (in mM): glucose, 10.1; NaCl, 115.5; $NaHCO_3$, 21.9; KCl, 4.6; NaH_2PO_4, 1.1; $CaCl_2$, 2.5 and $MgSO_4$, 1.2 (pH 7.3-7.4). The Krebs solution (37 ± 1 ºC) was bubbled with carbogen (95% O_2 and 5% CO_2). NANC conditions were obtained by adding phentolamine, propranolol and atropine (1 μM) to the Krebs solution to block α- and β-adrenoceptors and muscarinic receptors.

The following drugs were used: nifedipine, apamin, N^{ω}-nitro-L-arginine (L-NNA), α,β-methyleneadenosine 5´-triphosphate lithium salt (α,β-meATP), adenosine 5'-[β-thio]diphosphate trilitium salt (ADPβS), β-nicotinamide adenine dinucleotide hydrate (β-NAD), adenosine 5'-diphosphate sodium salt (ADP), adenosine 5´-triphosphate (ATP), adenosine 5'-diphosphoribose sodium salt (ADP-ribose), uridine-5'-triphosphate (UTP), uridine diphosphate glucose (UDP-glucose), phentolamine, oxadiazolo[4,3-α]quinoxalin-1-one (ODQ), nifedipine, atropine sulphate (Sigma Chemicals, St. Louis, USA), propranolol, (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), 8,8'-[carbonyl*bis*(imino-3,1-phenylenecarbonylimino)]*bis*-1,3,5-naphthalene-trisulphonic acid hexasodium salt (NF023), Pyridoxalphosphate-6-azophenyl-2',5'-disulfonic acid tetrasodium salt (iso-PPADS), 8,8'-[Carbonyl*bis*[imino-3,1-phenylenecarbonylimino(4-fluoro-3,1-phenylene)carbonylimino]]*bis*-1,3,5-naphthalenetrisulfonic acid hexasodium salt (NF157), ω-conotoxin GVIA, 6-N,N-Diethyl-D-β,γ-dibromomethyleneATP trisodium salt (ARL67156) (Tocris, Bristol, UK), tetrodotoxin (TTX) (Latoxan, Valence, France). 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

The effect of drugs on spontaneous contractility was calculated as a percentage of inhibition from the initial AUC, being 0% when no effect on spontaneous motility was observed and 100 % when a total inhibition of spontaneous motility was measured after drug administration. These data were used to calculate concentration-response curves (Constrains 0=bottom: no effect and 100=top total inhibition) using non-linear regression. EC_{50} (concentration that causes 50% reduction of spontaneous motility) and Hill slope were estimated in the absence and presence of the antagonist.

Normalization of EFS-induced effects was performed by calculating the percentage of control mechanical activity (previous AUC). In this case, 0% represents no effect of the antagonist and 100% represents a total reversion of EFS-induced inhibition of mechanical activity. A value higher than 100% indicates an excitatory response.

Differences in the amplitude of the IJPf before and after incubation with the antagonist were compared by One-Way ANOVA followed by Bonferroni post-hoc test. One-Way or Two-Way ANOVA were used to evaluate the effect of the different antagonists on inhibition of spontaneous motility induced by the purinergic agonists at single or cumulative doses and to evaluate the effect of the different antagonists on the inhibition of spontaneous motility evoked by EFS.

Data are expressed as mean ± S.E.M., and statistical significance was considered when $p < 0.05$. "n" values indicate the number of samples from different animals or patients. Statistical analysis and curve fit were performed with GraphPad Prism 6.00, GraphPad Software, San Diego, California, USA.

RESULTS

Effects of purine receptor antagonists on EFS-induced effects in the rat colon.

Rat colonic strips devoid of both mucosa and submucosa layers displayed rhythmic spontaneous contractions. In NANC conditions, EFS (5 Hz, 30 V, 0.3 ms, 2 min) caused complete inhibition of spontaneous contractions. Inhibition of spontaneous motility by EFS was recorded in the presence of L-NNA. Under these experimental conditions, cumulative concentrations of MRS2500 caused a concentration-dependent inhibition of EFS-induced effects (n=5) (Figure 1). In fact, an excitatory response was recorded at 1 µM of MRS2500. The P2X antagonists iso-PPADS (n=5) and NF023 (n=5) up to 1 µM did not modify EFS-induced inhibition of contractions or IJPf whereas at higher concentrations (10 µM) iso-PPADS partially reduced both of them. None of the purine receptor antagonists tested modified spontaneous motility in the presence of L-NNA (not shown).

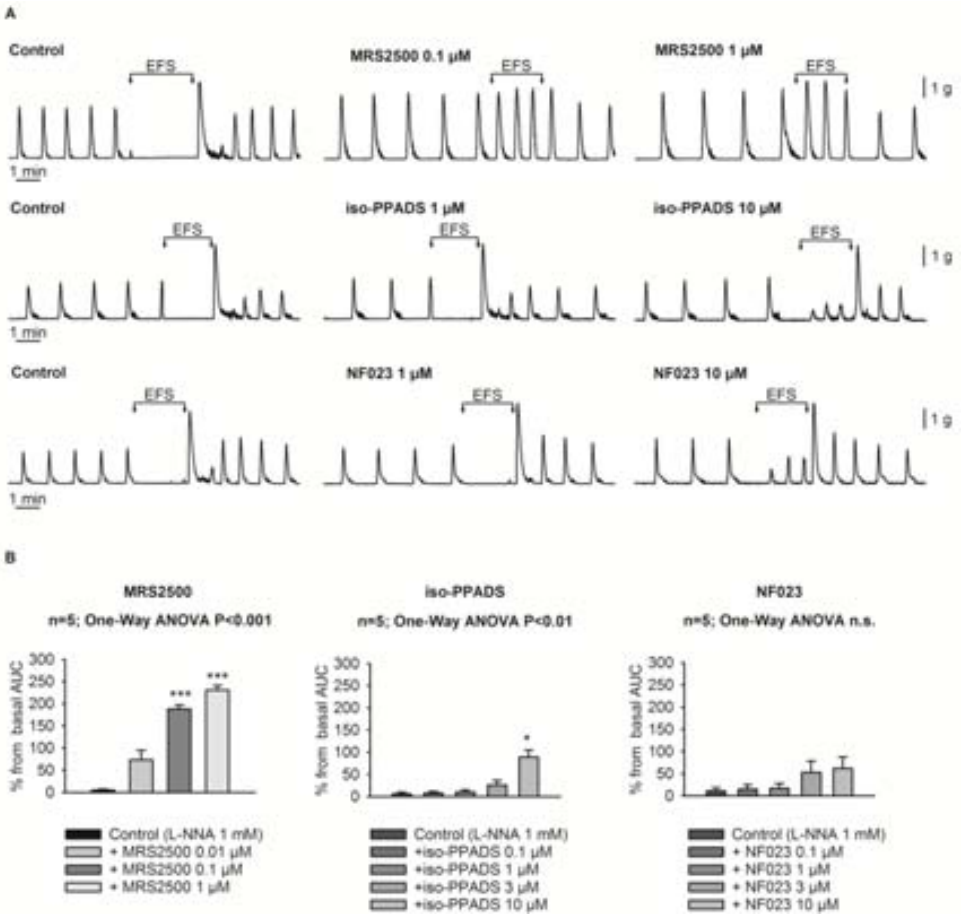

Figure 1. Mechanical recordings (A) and histograms (B) showing the high potency of the $P2Y_1$ receptor antagonist MRS2500 (0.01-1 µM) compared to iso-PPADS (0.1-10 µM) and NF023 (0.1-10 µM) on the reduction of the inhibition of the spontaneous contractility induced by EFS (control: L-NNA 1 mM in NANC conditions). Data higher than 100% of the initial AUC value (observed with MRS2500) represent a contractile response. Data are expressed as mean ± S.E.M. One-Way ANOVA was performed followed by a Bonferroni's multiple comparison test (* P<0.05, ***P<0.001; compared to control).

In the presence of L-NNA 1 mM, EFS elicited a purinergic IJPf. As previously described (Grasa et al., 2009), MRS2500 (n=4) concentration-dependently reduced the IJPf amplitude (IC_{50} =14 nM; $logIC_{50}$= -7.9±0.1) (Figure 2). NF023 (n=4) and iso-PPADS (n=4) did not significantly modify the response up to 3 µM, whereas at the highest concentration tested (10 µM) a partial reduction of the amplitude of the IJPf was observed (NF023 37.6 ± 8.4 %; iso-PPADS 26.8 ±4.1 % from control IJPf amplitude; Figure 2). Notice that in this tissue MRS2500 1 µM completely abolished EFS-induced cessation of contractions and IJPf, whereas at equivalent concentrations iso-PPADS and NF023 did not modify EFS-induced inhibition of contractions or IJPf.

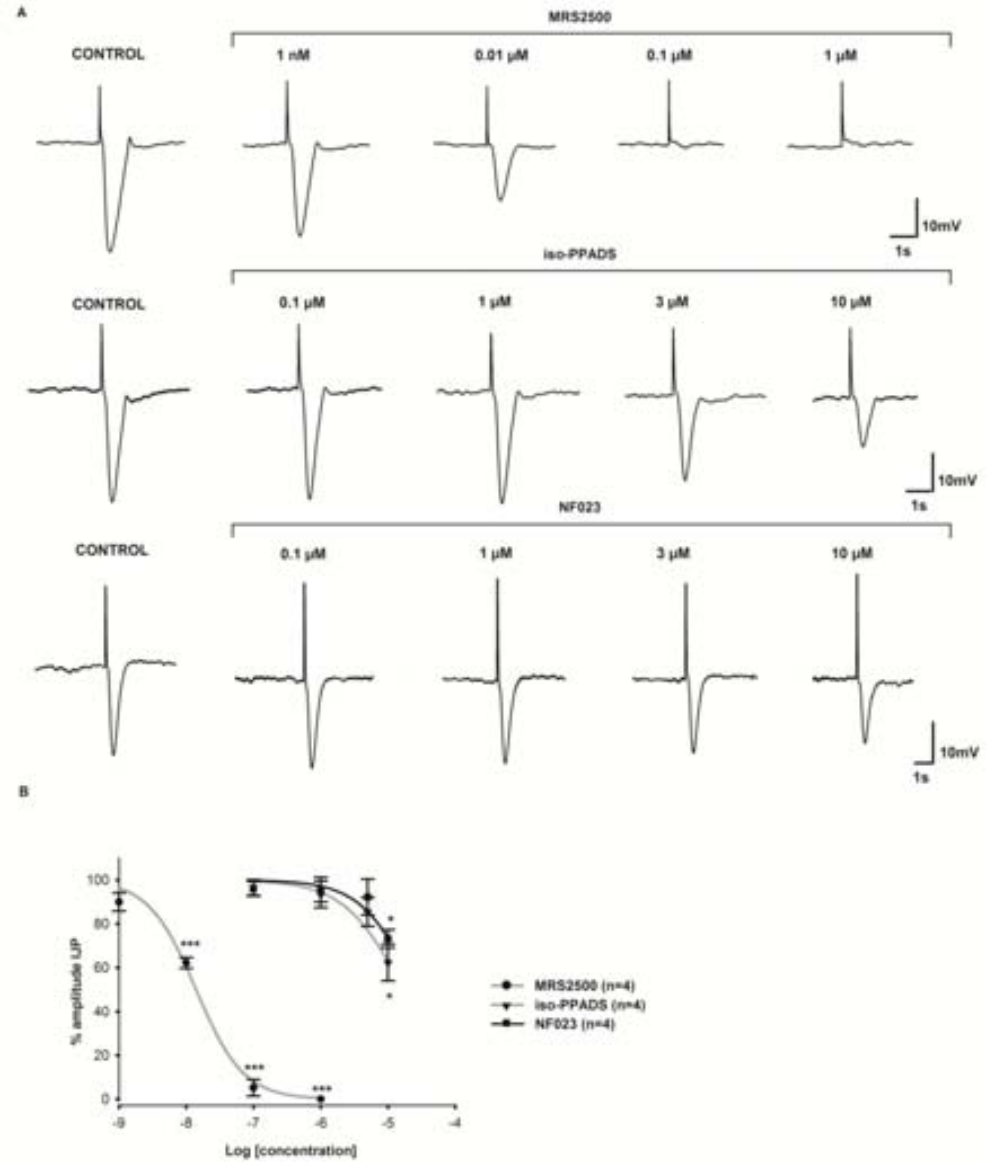

Figure 2. Intracellular microelectrode recordings (A) and graphical plot (B) showing the high potency of $P2Y_1$ receptor antagonist MRS2500 (1 nM-1 µM) (previously published in Grasa et al., 2009) compared to iso-PPADS (0.1-10 µM) and NF023 (0.1-10 µM) on the IJP induced by EFS. One-Way ANOVA was performed followed by a Bonferroni's multiple comparison test (* P<0.05, ***P<0.001; compared to the control IJP amplitude normalised to 100).

Effect of α,β-meATP on rat colonic contractions.

α,β-meATP (10 μM) inhibited spontaneous contractions (98.9±1.1%, n=8). This effect was also observed in the presence of TTX 1 μM (91.0±2.8 %, n=12), L-NNA 1 mM (90.5±4.8 %, n=8) or ODQ 10 μM (82.0±8.5%, n=5) suggesting that α,β-meATP is acting post-junctionally and the effect is not due to pre-junctional release of NO. In the presence of TTX and after a 15-min preincubation with MRS2500 (1 μM), α,β-meATP effect was antagonized, being reduced to 3.1±5.1 % (Figure 3). In contrast, tissue incubation with iso-PPADS or NF023 at 1 μM did not reduce the effect of α,β-meATP (iso-PPADS: 87.7±3.5%, n=6; NF023: 86.1±9.6%; n=5) (Figure 3). At higher concentrations of both antagonists (10 μM), EFS-induced inhibition of contractions and IJPf were partially reduced (Figure 1 and 2) and the effect of α,β-meATP was also significantly diminished (iso-PPADS: 37.3±5.0 %, n=5; NF023: 51.7±4.6%, n=5) (Figure 3). Similar results were obtained when the same protocol was performed in presence of L-NNA 1 mM (Figure 3). Consistent with the effect observed on contractility, α,β-meATP (10 μM) caused smooth muscle hyperpolarization (-7.9±2.1 mV, n=3) that was blocked by MRS2500 1 μM (n=2) but not by NF023 1 μM (n=3) (not shown).

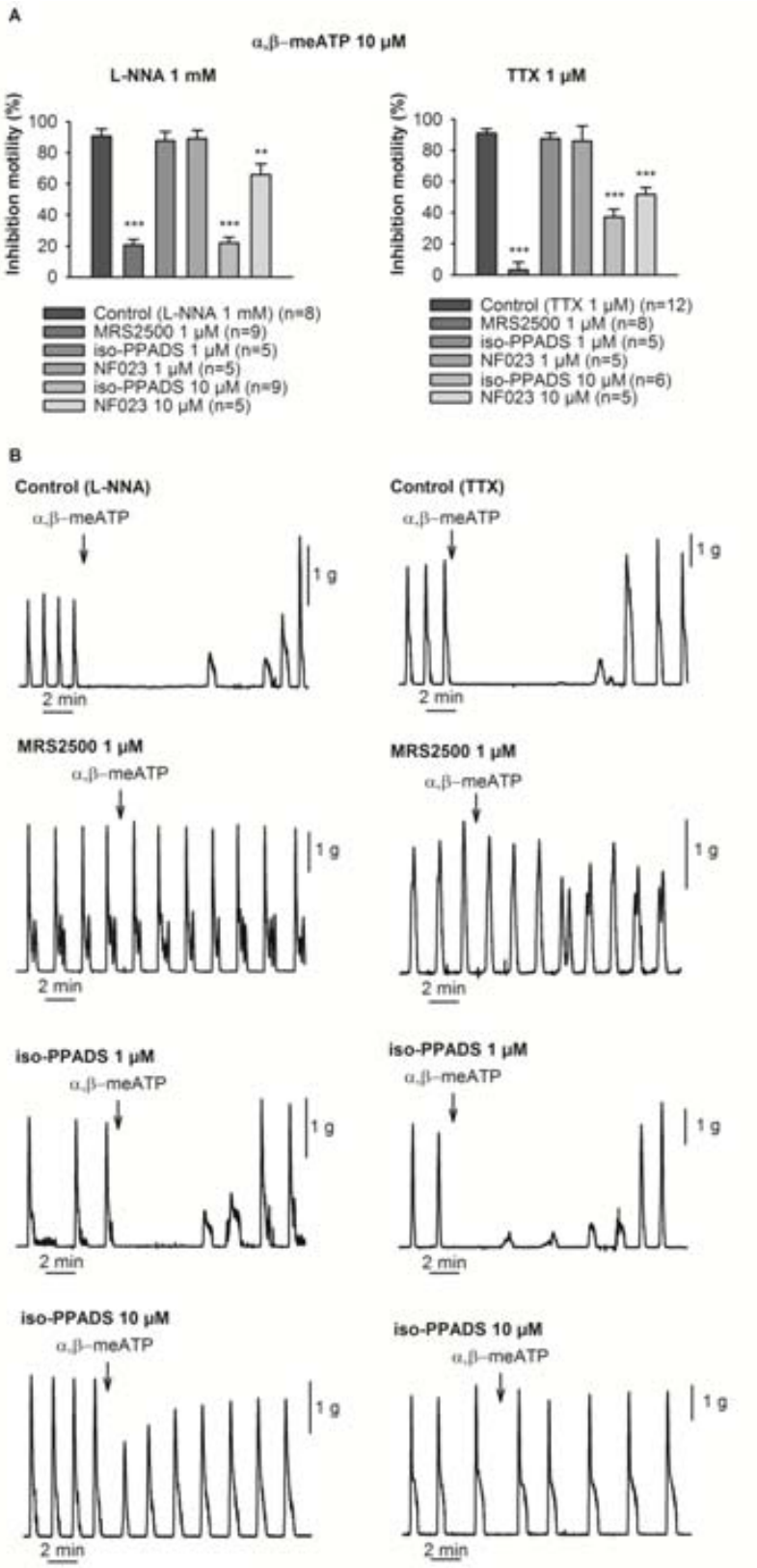

Figure 3. Histograms (A) and mechanical recordings (B) showing the effect of α,β-meATP 10 μM on rat colonic spontaneous contractions. Experiments were performed in the presence of L-NNA 1 mM (left) or TTX 1 μM (right). Notice that α,β-meATP effect was blocked by MRS2500 1 μM and concentration up to 10 μM of iso-PPADS and NF023 was needed to partially reduce α,β-meATP inhibitory effect. One-Way ANOVA was performed followed by a Bonferroni's multiple comparison test (** P<0.01, ***P<0.001).

Comparison between α,β-meATP and other purines on rat colonic contractility.

According to our previous results, we selected 1 μM concentration of the different antagonists (MRS2500, iso-PPADS and NF023) to characterize the effect of exogenous addition of purines. Experiments were performed in the presence of L-NNA (1 mM) to avoid any possible pre-junctional NO release. In these conditions, cumulative addition of α,β-meATP (0.01, 0.1, 1, 3, 10 μM) concentration-dependently inhibited spontaneous motility (Table 1) reaching 92.1±3.9 % of inhibition at 10 μM. The reduction of spontaneous motility induced by α,β-meATP was abolished by previous incubation with MRS2500 1 μM (6.1±3.8% at 10 μM (n=5). Tissue incubation with apamin 1 μM, a small conductance calcium-activated potassium channels (sKca) inhibitor, also abolished the effect induced by α,β-meATP, reaching 13.6±6.0% at 10 μM. α,β-meATP concentration-response curves were unaffected by iso-PPADS or NF023 both at 1 μM (Figure 4, Table 1).

Table 1. Table showing the n values, EC_{50}, $logEC_{50}$, hill slope and P value of the different protocols performed in rat colonic strips with the different purinergic agonists and antagonists tested.

Purinergic agonist	Antagonist	n	EC_{50} (μM)	$LogEC_{50}$ ± S.E.M.	Hill slope	P value
α,β-meATP	Control	6	2.7	-5.6±0.1	1.4±0.2	
	MRS2500 1 μM	5	ND	ND	ND	<0.0001
	Apamin 1 μM	5	ND	ND	ND	<0.0001
	iso-PPADS 1 μM	5	3.5	-5.5±0.1	1.8±0.4	ns
	NF023 1 μM	5	4.7	-5.3±0.1	1.1±0.4	ns
ATP	Control	5	27.9	-4.6±0.1	1.0±0.1	
	MRS2500 1 μM	5	49.8	-4.3±0.1	1.0±0.2	ns
	Apamin 1 μM	5	86.2	-4.1±0.1	0.9±0.2	0.0114
	iso-PPADS 1 μM	5	37.3	-4.4±0.1	1.0±0.1	ns
ADP	Control	5	72.5	-4.1±0.1	0.9±0.1	
	MRS2500 1 μM	5	59.6	-4.2±0.1	0.8±0.1	ns
	Apamin 1 μM	5	105.2	-4.0±0.1	1.0±1.4	ns
	iso-PPADS 1 μM	5	32.1	-4.5±0.1	0.9±0.1	ns
β-NAD	Control	7	74.1	-4.1±0.1	0.9±0.1	
	MRS2500 1 μM	5	48.4	-4.3±0.2	0.8±0.2	ns
	Apamin 1 μM	5	69.8	-4.2±0.1	0.7±0.1	ns
	iso-PPADS 1 μM	5	23.4	-4.6±0.1	1.1±0.2	ns
ADP-ribose	Control	6	34.9	-4.5±0.1	1.3±0.2	
	MRS2500 1 μM	5	35.4	-4.5±0.1	1.0±0.3	ns
	Apamin 1 μM	5	37.3	-4.4±0.2	0.5±0.2	ns
	iso-PPADS 1 μM	5	67.9	-4.2±0.1	1.6±0.5	ns

Notice that α,β-meATP effect was totally abolished with previous incubation with MRS2500 and apamin. ATP effect was shifted by previous incubation with apamin. Data are expressed as mean±S.E.M. P value was obtained by using Two-Way ANOVA comparing the response obtained with each antagonist to control conditions (ND: not determined).

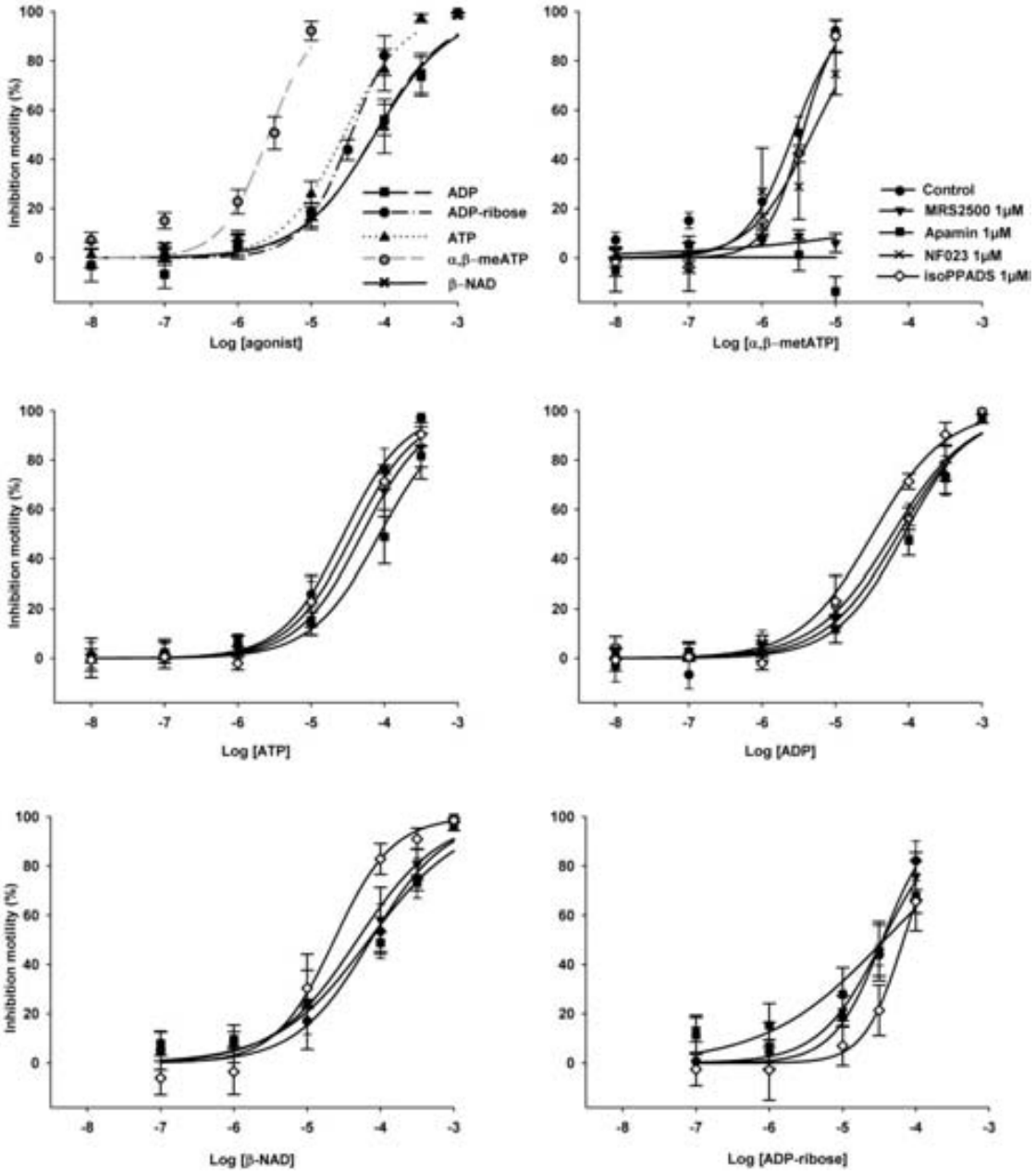

Figure 4. Graphical plots showing the effect of cumulative concentrations of α,β-meATP, ATP, ADP, β-NAD and ADP-ribose on the inhibition of spontaneous motility in the rat colon. Concentration-response curves were performed in the presence of L-NNA 1 mM (control) and after 15-min pre-incubation with MRS2500, apamin, iso-PPADS and NF023. Notice that the inhibition of spontaneous motility induced by α,β-meATP was blocked by MRS2500 and apamin both at 1 μM. Two-Way ANOVA was performed to compare the response between control and with pre-incubation with the different antagonists (Table 1).

Other purine receptor agonists tested (ATP, ADP, β-NAD and ADP-ribose) also inhibited spontaneous motility in a concentration-dependent manner (Figure 4). The rank of potency was: α,β-meATP > ATP > ADP-ribose > ADP ≈ β-NAD. No major differences in EC_{50} were observed between the four less potent compounds and in these cases incubation with 1 μM MRS2500 or iso-PPADS did not modify the responses. Apamin slightly shifted the concentration response curve of ATP (0.01, 0.1, 1, 10, 100, 300 μM) to the right (Figure 4, Table 1). UTP and UDP-glucose effects on spontaneous contractions were also evaluated; neither UTP nor UDP-glucose (both at 100 μM) elicited any inhibitory effect on rat colonic spontaneous contractions. As the stable analogue α,β-meATP mimicked the pharmacological profile of the endogenous purine and ATP did not, some experiments were performed with previous incubation (20 min) with ARL67156 to try to avoid ATP metabolism by ectonucleotidases. ARL67156 100 μM decreased spontaneous motility (49.7±7.4 % from control AUC, n=5). In the presence of ARL67156 (100 μM) ATP inhibitory effects were not modified and tissue incubation with MRS2500 1 μM did not antagonize ATP effects (n=5). The inhibition of spontaneous motility caused by EFS (in the presence of L-NNA, n=5) was not modified by ARL67156.

Purine receptor antagonists and EFS-induced effects in the human colon

Human colonic strips devoid of mucosa displayed rhythmic spontaneous contractions. In NANC conditions, EFS (2 Hz, 50 V, 0.4 ms, 2 min) caused almost a total cessation of spontaneous contractions. L-NNA 1 mM partially reduced the inhibitory effect induced by EFS. In these experimental conditions, the addition of either MRS2500 1 μM or apamin 1 μM, blocked the inhibitory effect of EFS. It is important to notice that an excitatory effect was recorded during EFS in the presence of either MRS2500 or apamin. On the contrary, neither the specific $P2Y_{11}$ antagonist NF157 nor the P2X antagonist iso-PPADS (both at 10 μM) modified the EFS-induced inhibition of spontaneous contractions (Figure 5).

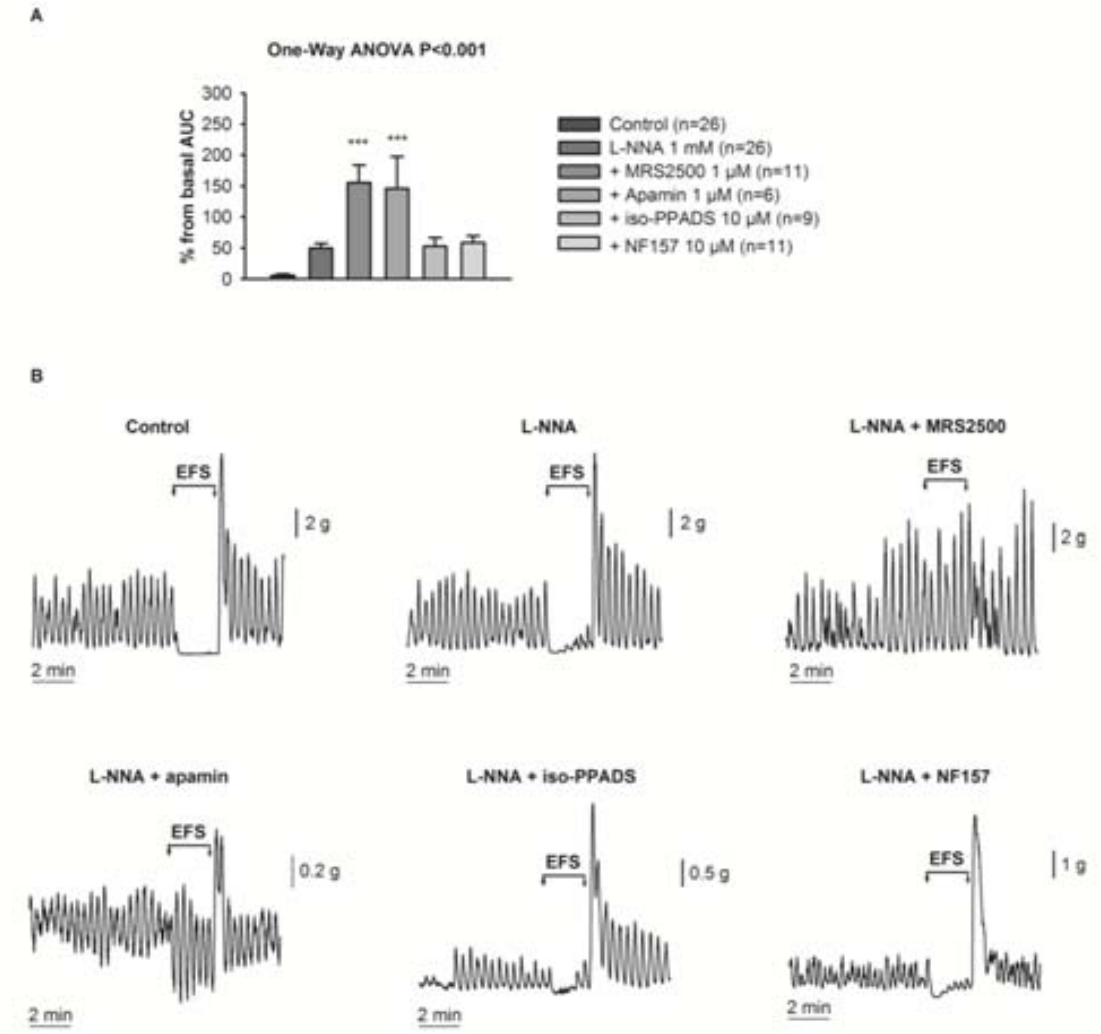

Figure 5. Histogram (A) and mechanical recordings (B) showing the effect of the $P2Y_1$ antagonist MRS2500 (1 μM) and the sKca blocker apamin (1 μM) compared to iso-PPADS (10 μM) and NF157 (10 μM) on the inhibition of the spontaneous activity induced by EFS in human colonic strips. L-NNA partially reduced EFS-induced inhibition of contractions and both MRS2500 and apamin added on top totally reversed the reduction of spontaneous motility induced by EFS (a slight contractile response with values higher than 100 % was often observed). In contrast NF157 (a $P2Y_{11}$ antagonist) and iso-PPADS (10 μM) did not inhibit the non-nitrergic EFS-induced inhibition of mechanical activity. One-Way ANOVA was performed followed by a Bonferroni's multiple comparison test (***P<0.001, compared to data obtained with L-NNA).

Effect of α,β-meATP on human colonic motility.

α,β-meATP 10 μM reduced spontaneous contractility in the human colon (70.2±14.8 % from basal AUC, n=3). In the presence of TTX 1 μM, α,β-meATP 10 μM inhibited spontaneous motility a 59.7±8.2 % of control AUC (n=5). The $P2Y_1$ antagonist MRS2500 1 μM significantly antagonized the inhibitory effect of α,β-meATP (4.4±9.9 %; n=5). In contrast, preferential P2X blockers such as iso-PPADS 10 μM (n=5) or NF023 10 μM (n=4) did not modify α,β-meATP

effect (Figure 6). Incubation with the ectonucleotidase inhibitor ARL67156 100 µM partially reduced spontaneous contractions (42.8±10.5% from control AUC, n=4) but did not modify the inhibition of contractions induced by EFS (n=3).

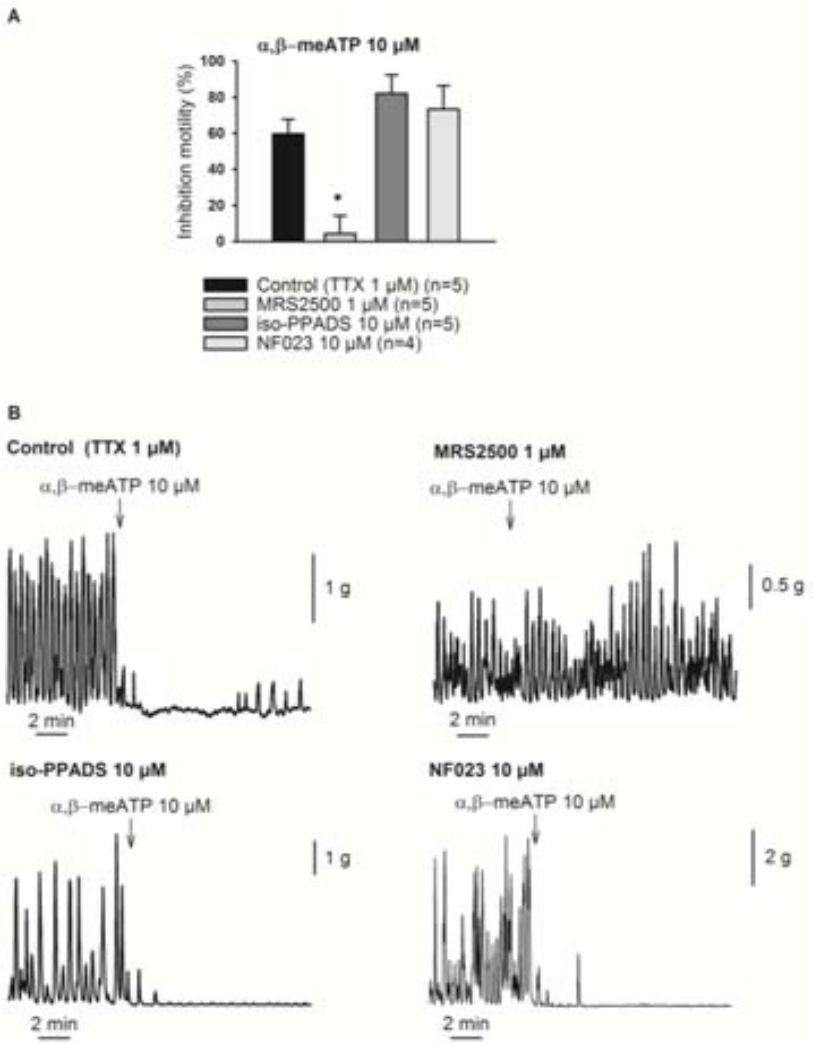

Figure 6. Histograms (A) and mechanical recordings (B) showing that the effect of α,β-meATP 10 µM (in the presence of TTX 1µM) on spontaneous contractility of human colonic preparations can be reduced with tissue pre-incubation with the $P2Y_1$ antagonist MRS2500 1µM but not with iso-PPADS or NF023 (10 µM). One-Way ANOVA was performed followed by a Bonferroni's multiple comparison test (* P<0.05, compared to control).

Cumulative concentration-response curve of α,β-meATP (0.1, 1, 10, 30 µM) was performed in the presence of L-NNA (1 mM). α,β-meATP (n=6) caused a concentration-dependent reduction of spontaneous contractions reaching 87.7±6.8 % of inhibition at 30 µM. This inhibitory effect was abolished by previous incubation with MRS2500 1 µM and apamin 1 µM. However, α,β-meATP inhibition was unaffected by the P2X antagonist iso-PPADS 10 µM, reaching an inhibitory effect of 83.6±5.7 % at 30 µM (Figure 7, Table 2).

Table 2. Table showing the n values, EC_{50}, $logEC_{50}$, hill slope and P value of the different protocols performed in human colonic preparations with the different purinergic agonists and antagonists tested.

Purinergic agonist	Antagonist	n	EC_{50} (μM)	$LogEC_{50}$ ± S.E.M.	Hill slope	P value
α,β-meATP	Control	6	4.4	-5.4±0.1	0.9±0.2	
	MRS2500 1 μM	7	ND	ND	ND	<0.0001
	Apamin 1 μM	7	ND	ND	ND	<0.0001
	iso-PPADS 10 μM	6	8.5	-5.1±0.2	0.9±0.3	ns
ATP	Control	6	123.7	-3.9±0.2	0.7±0.2	
	MRS2500 1 μM	9	370.9	-3.4±0.2	0.6±0.2	ns
	Apamin 1 μM	6	90970	-1.0±3.2	0.3±0.3	0.0028
	iso-PPADS 10 μM	6	450.9	-3.4±0.6	0.5±0.4	ns
ADP	Control	5	272.8	-3.6±0.1	1.0±0.3	
	MRS2500 1 μM	6	323.8	-3.5±0.2	0.7±0.3	ns
	Apamin 1 μM	5	1180	-2.9±0.3	0.6±0.3	ns
	iso-PPADS 10 μM	5	422.1	-3.4±0.2	0.6±0.2	ns
β-NAD	Control	5	5780	-2.2±0.1	2.3±1.1	
	MRS2500 1 μM	6	5080	-2.3±0.1	3.0±1.6	ns
	Apamin 1 μM	5	6390	-2.2±0.1	2.5±0.9	ns
	iso-PPADS 10 μM	5	5270	-2.3±0.1	1.9±0.6	ns
ADPβS	Control	6	1.1	-6.0±0.3	0.9±0.4	
	MRS2500 1 μM	7	9.3	-5.0±0.2	1.5±1.2	<0.0001
	Apamin 1 μM	6	2590	-2.6±1.4	0.3±0.1	<0.0001
	iso-PPADS 10 μM	5	5.5	-5.3±0.6	0.4±0.2	ns

Notice that α,β-meATP effect was totally abolished with previous incubation with MRS2500 and apamin. The response with the preferential P2Y agonist ADPßS was reduced with MRS2500 and apamin. ATP effect was also partially apamin sensitive. Data are expressed as mean±S.E.M. P value was obtained by using Two-Way ANOVA comparing the response obtained with each antagonist to control conditions (ND: not determined).

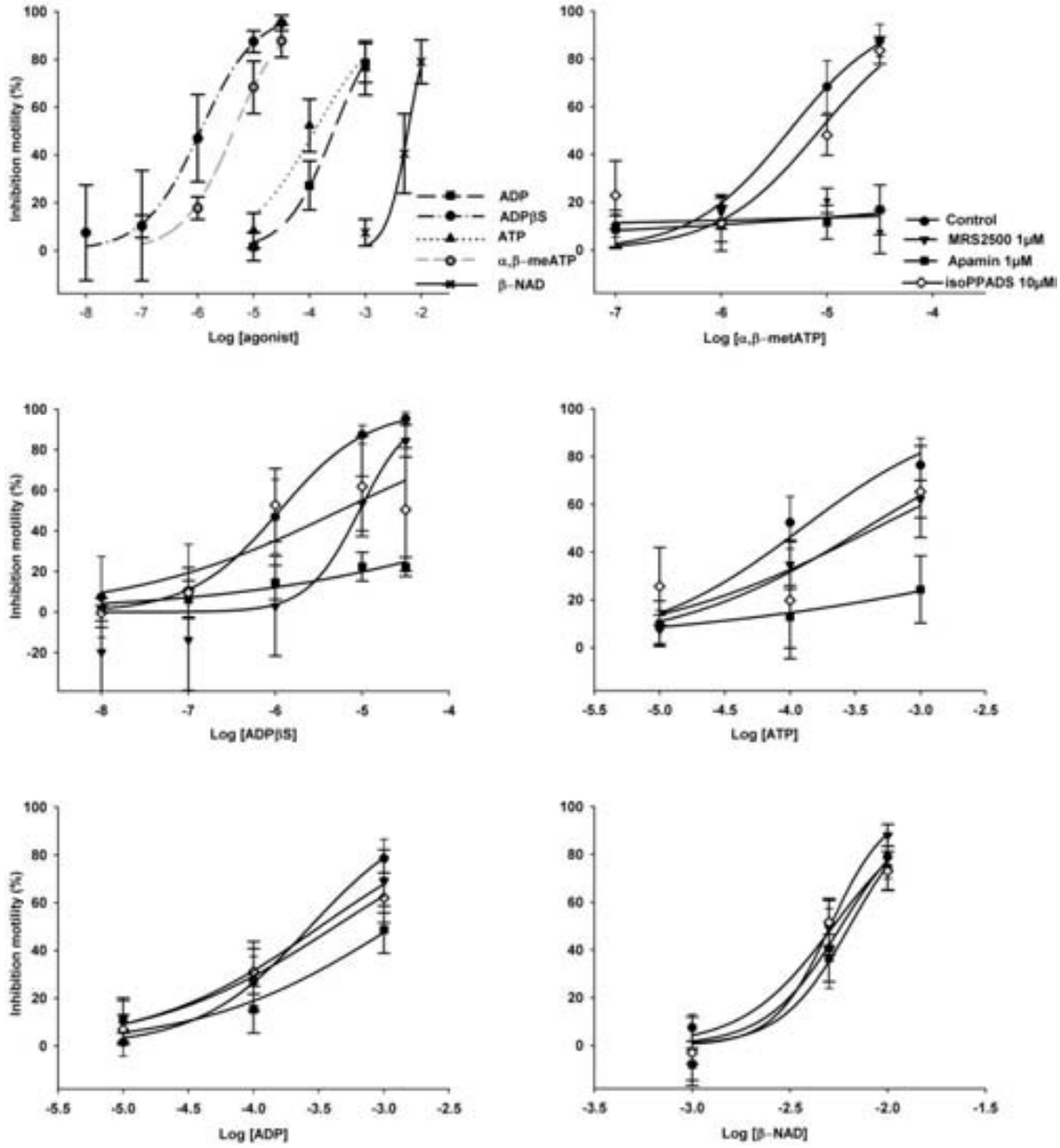

Figure 7. Graphical plots showing the effect of cumulative concentrations of α,β-meATP, ATP, ADP, β-NAD and ADPβS in the % of inhibition of spontaneous contractions in human colonic preparations. Concentration-response curves were performed in presence of L-NNA 1 mM. Two-Way ANOVA was performed to compare the effects of every agonist in control conditions and after pre-incubation with the different antagonists (Table 2).

Other purine receptor agonists were tested and most of them were able to significantly reduce colonic spontaneous contractions. The rank of potency of the active compounds was: ADPβS > α,β-meATP > ATP > ADP> β-NAD>ADP-ribose . ADP-ribose (0.1, 1, 10, 100 μM) inhibited spontaneous motility in only four out of eleven samples. In the samples in which ADP-ribose reduced contractility, the percentage of inhibition was only 33.4±5.0 % at the highest concentration tested (100 μM). MRS2500 1 μM shifted to the right the concentration-response curve obtained with ADPβS (0.01, 0.1, 1, 10, 30 μM). Apamin 1 μM antagonized the inhibitory effect elicited by cumulative concentration-response curves of ADPβS and ATP (10, 100, 1000 μM). None of the responses were antagonized by iso-PPADS 10 μM (Figure 7, Table 2). UDP-glucose and UTP (both at 100 μM) effects on contractility were also tested. UDP-glucose did not elicit any inhibitory effect and UTP reduced spontaneous contractility in five out of thirteen samples with a low percentage of inhibition (49.9±13.4 % control; 31.0±11.3 % in the presence of TTX 1 μM).

DISCUSSION

At the moment there is an agreement about the crucial role of $P2Y_1$ receptors in purinergic relaxation (King, 2012;Goyal *et al.,*2013). In the present work we confirm that non-selective P2X antagonists such as iso-PPADS and the preferential $P2X_1$ antagonist NF023 do not inhibit purinergic neuromuscular transmission unless high concentrations of the antagonists are used (10 μM in the rat colon). PPADS, a preferential P2X antagonist, reduces purinergic relaxation of the rat pylorus (Ishiguchi *et al.,* 2000) and ileum (Storr *et al.,* 2000) at a concentration of 30 μM. This response is possibly due to an unspecific effect of these antagonists on $P2Y_1$ receptors. Comparatively, MRS2500 totally abolished EFS-induced inhibition of contractions and IJPf at 1 μM. Moreover, in the presence of MRS2500 1 μM an excitatory response was recorded during EFS both in the human and rat colon. This result suggests that when the purinergic response is abolished, the predominant response is excitatory, possibly due to tachykinins (Auli *et al.,* 2008) when NANC conditions are used (present work). Accordingly, a clear difference between the effect of MRS2500 and the other purinergic antagonists tested can be clearly established.

Other receptors have been postulated to be related to the purinergic pathway. The "orphan" G protein-coupled receptor GPR17 is structurally related to P2Y receptors and is MRS2179-sensitive. UDP-glucose is considered a GPR17 receptor ligand (Temporini *et al.,*2009;Pugliese *et al.,*2009) but it did not modify spontaneous contractility. UTP, a $P2Y_2$ and $P2Y_4$ agonist, at a concentration of 100 μM did not cause any effect in rat preparations and its inhibitory effect in human was weak and infrequent. $P2Y_{11}$ receptor is another possible candidate to mediate smooth muscle relaxation (King & Townsend-Nicholson, 2008). However, the $P2Y_{11}$ blocker NF157 up to 10μM did not modify EFS-induced inhibition of contractions in the human colon. Consistent with a major role of $P2Y_1$ receptors mediating purinergic relaxation and similarly to previous data obtained in rodents (De Man et al., 2003;Grasa et al., 2009), the preferential $P2Y_1$ agonist ADPßS and the selective $P2Y_1$ agonist MRS2365 (Gallego *et al.,*2012) caused relaxation in the human colon, which was blocked by MRS2500 and substantially antagonized by apamin. We conclude that $P2Y_1$ receptors but not P2X receptors likely mediate neural mediated inhibitory responses in humans and rodents.

Another aim of this work was to study which purine better mimics endogenous responses. α,β-meATP is considered from a pharmacological point of view as a P2X agonist often used to discriminate between P2X and P2Y effects (De Man *et al.,*2003; Leon *et al.,*2006). Inhibitory effects induced by α,β-meATP were reduced by high concentrations of MRS2179

both in rat (3 to 30 μM) (Van Crombruggen et al.,2007) and human colon (10 μM) (Auli *et al.,*2008). However, at high concentration the selectivity of MRS2179 on $P2Y_1$ receptors is questionable (Van Crombruggen et al., 2007;Zizzo et al.,2007;Auli et al.,2008;McDonnell *et al.,*2008). Here we show that 1- MRS2500 1 μM inhibited α,β-meATP effects, including the effect on resting membrane potential and spontaneous contractility, 2- α,β-meATP effects are unaffected by iso-PPADS or NF023 at concentrations that are not able to inhibit EFS-induced inhibition of contractions or IJPf, 3- α,β-meATP inhibitory effect is not due to nerve mediated TTX-sensitive responses leading to prejunctional release of inhibitory transmitters such NO and 4- α,β-meATP inhibitory effects are apamin sensitive, therefore involving sKca channels. These findings are consistent with a direct effect of α,β-meATP on post-junctional $P2Y_1$ receptors and confirm the atypical response previously reported (Giaroni *et al.,*2002). However, at least in the rat colon, 10 μM iso-PPADS and NF023 partially reduced α,β-meATP inhibitory effect which might be consistent with an effect on P2X receptors located in either smooth muscle (Storr *et al.,*2000) or nerve varicosities that cause purine release (King & Townsend-Nicholson, 2008). The most attractive hypothesis is that α,β-meATP is releasing a purine which in turn acts on $P2Y_1$ receptors causing smooth muscle inhibition of spontaneous motility. In fact we cannot totally reject this hypothesis at least in the rat colon. However, high concentrations of P2X antagonists caused a certain inhibitory effect on the $P2Y_1$ mediated IJPf and therefore the window of concentrations is too small to have a certain answer. In the human colon iso-PPADS and NF023 at 10 μM did not inhibit α,β-meATP effects which is apparently not consistent with an effect of α,β-meATP on P2X receptors.

Nowadays, the debate about the nature of the purinergic NT is still open (Goyal *et al.,*2013). ATP has been the main candidate for years (Burnstock *et al.,* 1970;Burnstock, 2008). ATP is stored in nerve varicosities that contain the SCL17A9 transporter (Chaudhury *et al.,*2012) and is released from enteric neurons (White & Leslie, 1982). β-NAD (Hwang *et al.,*2011) and its metabolite ADP-ribose (Durnin *et al.,*2012) have been recently proposed as purinergic NTs. Our results do not solve discrepancies between studies; however, it is important to take into account the following ideas.

α,β-meATP is a synthetic analogue of ATP, resistant to ectonucleotidase metabolism (Van Crombruggen *et al.,* 2007). Exogenous addition of ATP is quickly metabolized to adenosine (Duarte-Araujo *et al.,* 2009) and the putative effect of ATP might be due to multiple purine metabolites including ADP and adenosine. Nevertheless, exogenously added adenosine does not cause smooth muscle hyperpolarization in the human colon (Gallego *et al.,* 2008a).

ATP effects are partially apamin-sensitive both in rat and human colon (present study) suggesting that exogenous ATP partially mimics the NT. In our study, an important difference between α,β-meATP and ATP effect is that the first one is blocked by MRS2500 and the second one is not. ATP effects were studied after incubating the tissue with the ectonucleotidase inhibitor ARL67156 (100 μM). In the presence of ARL67156 a decrease in spontaneous motility was observed both in human and rat colon. We can speculate that inhibition of ectonucleotidase can cause accumulation of active purines at the neuromuscular junction inhibiting spontaneous contractions. In the presence of ARL67156, EFS-induced inhibition of spontaneous motility persisted, which is consistent with a lack of effect of ARL67156 on the IJPf (Matsuda *et al.,* 2004). Our results demonstrate that in the rat colon ATP effects persist in the presence of ARL67156 and the response to ATP is still MRS2500 insensitive. Accordingly, under putative ectonucleotidase inhibition the pharmacological response to ATP was not modified. However, as the utility of ARL67156 to block ectonucleotidase activity is under discussion (Levesque *et al.,* 2007;Fei *et al.,* 2013) we cannot discard that the lack of effect of MRS2500 on ATP inhibitory effect is due to its degradation. Accordingly, our results suggest that ATP partially mimics the purinergic NT but the effect is not exclusively due to $P2Y_1$ activation.

High concentrations of β-NAD induced an MRS2500-sensitive slight hyperpolarization (4-6 mV) in human colonic tissue (Hwang *et al.,* 2011;Gallego *et al.,* 2011). In this study we confirm that β-NAD mechanical inhibitory responses are insensitive to MRS2500 (Gallego *et al.,* 2011), iso-PPADS or apamin, suggesting that β-NAD is targeting multiple receptors and pathways as it might also be the case for ATP/ADP. Similar results were obtained with ADP-ribose in the rat colon. In human tissue, high concentrations of ADP-ribose are needed to inhibit spontaneous motility and this inhibitory effect is not always observed. Our experiments cannot exclude that these compounds could contribute to purinergic neurotransmission. It is possible that exogenous addition of a mediator does not exactly mimic the effect elicited by its local release and specific activation of receptors located at the neuromuscular junction. According to this hypothesis, it has been shown that fibroblast like cells (PDGFRα+ cells) expressing $P2Y_1$ receptors and sKca channels are probably crucial cells mediating purinergic inputs (Kurahashi *et al.,* 2011). Differences in gene expression of purine receptors and channels in smooth muscle and PDGFRα+ cells (Peri *et al.,* 2013) might explain differences between local release and exogenous addition of purines. Unfortunately, there is still not a consensus about the role of ICC or PDGFRα+ cells as intermediates of nitrergic and purinergic inhibitory responses respectively (Goyal *et al.,* 2013). What is really interesting about our study

is that α,β-meATP perfectly mimics the endogenous response despite the presence of extrajunctional receptors that can also be activated by the compound, including P2X receptors in SMCs. What makes α,β-meATP so specific mimicking endogenous purinergic responses? Unfortunately our study does not have a definitive explanation for this question but both a direct effect on the receptor or an indirect effect due to release of a local mediator (see the discussion above) are possible but speculative.

The identification of molecules with potential effects on smooth muscle relaxation in whole-tissue is necessary to approach the treatment of motor disorders. Nowadays, NO donors are commonly used as sphincter relaxants. Therefore, $P2Y_1$ receptors are also possible future pharmacological targets to take into account. In this case, exogenously added compounds should be potent enough to elicit an inhibitory effect and able to mimic the physiological route of relaxation. This might be relevant if studies performed with animal models of inflammation lacking purinergic neuromuscular transmission (Strong *et al.,* 2010;Roberts *et al.,* 2012) have translation to human diseases.

We have designed a pharmacological approach to distinguish between endogenous NTs acting through $P2Y_1$ receptors and through other purine receptors. The purinergic endogenous response responsible for smooth muscle hyperpolarization and relaxation is mediated through $P2Y_1$ receptors and sKca channels in both human and rat colon. Based on this criteria, we have demonstrated that the synthetic ATP analogue α,β-meATP is the purine tested that better mimics the endogenous purinergic response both in the rat and human colon. We suggest that $P2Y_1$ receptors are a potential pharmacological target leading smooth muscle relaxation to treat spasticity in colonic motor disorders.

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CHAPTER 2

EP_2 and EP_4 receptors mediate PGE_2 induced relaxation in murine colonic circular muscle: pharmacological characterization

Pharmacological research. Under revision

ABSTRACT

Prostaglandin E_2 (PGE_2) is a regulator of gastrointestinal motility that might be involved in impaired motor function associated to gut inflammation. The aim of the present work is to pharmacologically characterise responses to exogenous and endogenous PGE_2 in the mouse colon targeting EP_2 and EP_4 receptors. Wild type (WT) and EP_2 receptor knockout (EP_2-KO) mice were used to characterise PGE_2 and butaprost (EP_2 receptor agonist) effects on smooth muscle resting membrane potential and myogenic contractility in circularly oriented colonic preparations**.** In WT animals, PGE_2 and butaprost concentration-dependently inhibited spontaneous contractions and hyperpolarized smooth muscle cells (SMCs). Combination of both EP_2 (PF-04418948 0.1 μM) and EP_4 receptor antagonists (L-161,982 10 μM) was needed to block both electrical and mechanical PGE_2 responses. Butaprost inhibitory responses (both electrical and mechanical) were totally abolished by PF-04418948 0.1 μM. In EP_2-KO mice, PGE_2 (but not butaprost) concentration-dependently inhibited spontaneous contractions and hyperpolarized SMCs. In EP_2-KO mice, PGE_2 inhibition of spontaneous contractility and hyperpolarization was fully antagonized by L-161,982 10 μM. In WT animals, EP_2 and EP_4 receptor antagonists caused a smooth muscle depolarization and an increase in spontaneous mechanical activity. PGE_2 responses in murine circular colonic layer are mediated by post-junctional EP_2 and EP_4 receptors. PF-04418948 and L-161,982 are selective EP_2 and EP_4 receptor antagonists that inhibit PGE_2 responses. These antagonists might be useful pharmacological tools to limit prostaglandin effects associated to dismotility in gut inflammatory processes.

INTRODUCTION

Prostaglandin E_2 (PGE_2) is widely produced in different organs from different species and plays a relevant role in several gastrointestinal (GI) functions (Sugimoto & Narumiya, 2007) including mucosal protection, secretion and motility (Dey *et al.*, 2006). Two isoforms of cyclooxygenase (COX) enzyme are responsible for prostaglandin (PG) synthesis: COX-1 and COX-2. In physiological conditions, the gut mucosa expresses high levels of COX-1 and low levels of COX-2 (Dey *et al.*, 2006). Despite that COX-2 is typically considered an inducible isoform highly expressed during the inflammatory state, a constitutive expression has been demonstrated in several GI tissues, including the murine proximal colon, where products derived from constitutive COX-2 seem to contribute to the tonic inhibition of the contractile activity of circular smooth muscle layer (Porcher *et al.*, 2004). Besides its physiological role, elevated production of PGE_2 induced by an up-regulated expression of COX-2 might participate in the impaired motility associated to pathophysiological processes such as inflammatory bowel disease or slow transit constipation (Dey *et al.*, 2006;Cong *et al.*, 2007).

PGE_2 targets four G protein-coupled (GPCR) EP receptors: EP_1, EP_2, EP_3 and EP_4. The signalling pathway triggered is different for each EP receptor subtype (Dey *et al.*, 2006). EP receptor expression varies depending on the tissue and the cell type (Sugimoto & Narumiya, 2007). According to their effects on smooth muscle, the four EP receptors can be classified in two groups: “relaxant” EP_2 and EP_4 receptors and “contractile” EP_1 and EP_3 receptors (Narumiya *et al.*, 1999). EP_2 and EP_4 receptor activation increases cyclic adenosine monophosphate (cAMP) and causes smooth muscle relaxation. Activation of EP_1 receptors induces calcium mobilization and they are considered “contractile” receptors. Finally EP_3 receptors, termed the “inhibitory” receptors, reduce cAMP levels and lead to contraction (Narumiya & FitzGerald, 2001). PGE analogues are usually not specific for an EP receptor with the exception of butaprost, which specifically binds to EP_2 receptors (Sugimoto & Narumiya, 2007). One of the difficulties to identify the receptor involved in PGE_2 response has been the lack of selective antagonists to discriminate between EP receptor subtypes. Recently, PF-04418948 has been developed as a potent and selective EP_2 receptor antagonist (af Forselles *et al.*, 2011;Birrell & Nials, 2011).

PGE_2 is considered an important regulator of GI motility (Dey *et al.*, 2006) and both contraction and relaxation have been observed after its exogenous addition. In circular smooth muscle from canine colon, PGE_2 causes both relaxant and contractile responses (Botella *et al.*, 1995). PGE_2 induced relaxation of the circular muscle and contraction of the longitudinal muscle has been reported in human, guinea-pig and rat small intestine (Bennett *et al.*, 1968b)

as well as in human colon (Bennett *et al.*, 1981). Another study has shown that PGE_2 contracts longitudinal smooth muscle in human colon through EP_1 receptors and both mouse ileum and colon through EP_1 and EP_3 receptors, whereas activation of EP_2 receptor by butaprost reduces the tension in the three preparations (Fairbrother *et al.*, 2011). PGE_2 increases the frequency of peristaltic contractions and evokes the appearance of ectopic sites of waves, an effect that is not observed in interstitial cells of Cajal (ICCs) deficient mice (Forrest *et al.*, 2009), suggesting a possible effect of PGE_2 on ICC function. PGE_2 causes nerve mediated contractions in the longitudinal layer of the rabbit small intestine through EP_1 and EP_3 receptors (Grasa *et al.*, 2006), which might participate in the generation of spontaneous contractions in the longitudinal layer of the rat colon (Iizuka *et al.*, 2014). This dual effect may be explained by different expression of EP receptors in different subclasses of enteric neurons, smooth muscle or ICC. Due to the different location of EP receptors and the lack of selective pharmacological tools, the receptors involved in these responses are not yet clearly established.

Accordingly, the aim of this paper was: 1- to investigate the effects of PGE_2 in circular colonic smooth muscle contractility and membrane potential, 2- to investigate the receptors mediating PGE_2 actions and 3- to determine the receptors involved in the effects of endogenously produced PGE_2. Different agonists (PGE_2 and butaprost) and selective antagonists of EP_2 (PF-04418948) and EP_4 (L-161,982) receptors were assessed in colonic tissue from both wild-type (WT) and EP_2 knockout mice (EP_2-KO). Our results may help to better understand the role of PGE_2 in the regulation of colonic motor activity, and hence, to contribute to the design of pharmacological therapeutic strategies for motor disorders associated to inflammatory gut processes.

MATERIALS AND METHODS

Mouse tissue preparation

EP_2-deficient mice (B6.129-*Ptger2*^{tm1Brey}/J Jackson stock number 004376; n=13) and littermate controls (WT, n=21) aged 12-16 weeks old from Jackson Laboratories (Bar Harbor, ME, US) were used. Animals were kept at a constant room temperature (19-21ºC) and humidity (60 %), with a lighting cycle of 12 h light/12 h dark and *ad libitum* access to water and food. Animals were killed by cervical dislocation. The colon was placed in carbogenated Krebs solution. The colon was opened along the mesenteric border and pinned to a Sylgard base with the mucosa facing upwards. Mucosa and submucosa layers were carefully removed and circular muscle strips were cut into 6 mm long and 2 mm wide. This procedure was approved by the Ethics Committee of the Universitat Autonoma de Barcelona.

Intracellular microelectrode recording

Muscle strips were pinned to the base of a Sylgard coated chamber with the circular muscle side up and continuously perfused with "Non-adrenergic, non-cholinergic" (NANC) Krebs solution. Strips were allowed to equilibrate for approximately 1 h before the beginning of the experiments. Circular smooth muscle cells (SMCs) were impaled with sharp glass microelectrodes filled with 3 M KCl (30-60 MΩ). Membrane potential was measured using standard electrometer Duo773 (WPI Inc., Sarasota, FL, USA). Tracings were displayed on an oscilloscope 4026 (Racal-Dana Ltd., Windsor, UK) and simultaneously digitalized (100 Hz) using PowerLab 4/30 system and Chart 5 software for Windows (all from ADInstruments, Castle Hill, NSW, Australia). Nifedipine (1 µM) was used to abolish the mechanical activity and obtain stable impalements. In order to study PGE_2 effects on colonic neurotransmission, both components of the inhibitory junction potential (IJP) were studied. To isolate the fast (IJPf, purinergic) and the slow IJP (IJPs, nitrergic), tissue was previously incubated with L-NNA 1 mM and MRS2500 1 µM respectively (Gil *et al.*, 2012). The purinergic component was characterised using single pulses of electrical field stimulation (EFS) (pulse duration 0.3 ms) at increasing voltages (8, 12, 16, 20, 24, 28, 32, 36, 40 V). To study the nitrergic component, the tissue was stimulated with EFS for 20 s (pulse duration 0.3 ms, 5 Hz and 28 V).

Mechanical studies

Strips were placed in 10 mL organ bath containing Krebs solution. Circularly-oriented strips were tied using 3/0 silk thread to an isometric force transducer (Harvard VF-1 Harvard

Apparatus Inc., Holliston, MA, USA) to measure spontaneous mechanical activity. Data were digitalized (25 Hz) using Data 2001 software (Panlab, Barcelona, Spain) coupled to an ISC-16 A/D card. A tension of 0.5 g was applied and the tissue was allowed to equilibrate for 1 h. After this period, strips displayed spontaneous phasic contractions. To estimate mechanical activity responses to drugs, the area under curve (AUC) of contractions from the baseline was measured before and after drug addition. AUC was expressed as grams per minute ($g \cdot min^{-1}$).

Solutions and drugs

Krebs solution (composition in mM: glucose, 10.1; NaCl, 115.5; $NaHCO_3$, 21.9; KCl, 4.6; NaH_2PO_4, 1.1; $CaCl_2$, 2.5 and $MgSO_4$, 1.2, pH 7.3-7.4) was maintained at 37 ± 1 ºC and bubbled with carbogen (95% O_2 and 5% CO_2). NANC conditions (phentolamine, propranolol and atropine all at 1 μM) were used in microelectrode experiments to properly characterise the effects on inhibitory neurotransmission.

The following drugs were used: nifedipine, N^{ω}-nitro-L-arginine (L-NNA), phentolamine, nifedipine, atropine sulphate**,** prostaglandin E_2 (PGE_2) (Sigma Chemicals, St. Louis, USA), propranolol, (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) (Tocris, Bristol, UK), tetrodotoxin (TTX) (Latoxan, Valence, France), butaprost, 1-(4-fluorobenzoyl)-3-[[(6-methoxy-2-naphthalenyl)oxy]methyl]-3-azetidinecarboxylic acid (PF-04418948), N-[[4'-[[3-butyl-1,5-dihydro-5-oxo-1-[2-(trifluoromethyl)phenyl]-4H-1,2,4-triazol-4-yl]methyl][1,1'-biphenyl]-2-yl]sulfonyl]-3-methyl-2-thiophenecarboxamide (L-161,982) (Cayman Chemical - Vitro S.A., Madrid, Spain). Stock solutions were made by dissolving drugs in distilled water except for PGE_2, butaprost, PF-04418948 and L-161,982 which were dissolved in dimethyl sulphoxide, nifedipine which was dissolved in 96% ethanol and L-NNA which was dissolved in Krebs solution by sonication.

Data analysis and statistics

Smooth muscle resting membrane potential (RMP) was measured after local administration of agonists (PGE_2 and butaprost) in the absence (control) and in the presence of EP_2 (PF-04418948) and EP_4 (L-161,982) antagonists. The effects of the antagonists on RMP were also studied. The amplitude of the IJPf and IJPs was measured before and after PGE_2 infusion.

To normalize results from mechanical studies, the effect of cumulative concentration-response curves of PGE_2 and butaprost was calculated as percentage of inhibition from initial

AUC. Accordingly, 0% represents no effect on spontaneous motility and 100 % indicates a total inhibition of spontaneous contractility. Concentration-response curves were calculated using non-linear regression, and $LogEC_{50}$ and Hill slope were estimated in the absence and presence of the antagonists with constraints fixed at 0 (bottom) and 100% (top). To determine the effect of PF-04418948 and L-161,982 on spontaneous contractions, the percentage of increase from initial AUC was also calculated.

Differences in the RMP before and after infusion of the different drugs were compared by Paired t-test or One-Way ANOVA followed by a Bonferroni's multiple comparison test. Paired t-test or Two-Way ANOVA was used to evaluate the effects of the different antagonists on the concentration-response curves of PGE_2 and butaprost and to evaluate the effects of PGE_2 on nitrergic and purinergic components of the neurotransmission.

Data are expressed as mean ± S.E.M., and statistical significance was considered when $P < 0.05$. "n" values indicate the number of samples from different animals. Statistical analysis and curve fit were performed with GraphPad Prism 5.00, GraphPad Software, San Diego, California, USA.

RESULTS

PGE_2 and butaprost inhibited spontaneous contractility in mouse colon by a direct action on smooth muscle.

As previously described, circularly oriented colonic strips displayed myogenic rhythmic spontaneous contractions (Domenech *et al.*, 2011). PGE_2 concentration-dependently inhibited spontaneous contractions in both WT (EC_{50}= 10.6 nM; n=8) and EP_2-KO mice (EC_{50}= 23.6 nM; n=9), an effect that was also observed after addition of the selective EP_2 agonist butaprost in WT mice (EC_{50}= 15.5 nM, n=5). Consistent with a selective effect on EP_2 receptors, butaprost did not induce any change in spontaneous contractions in EP_2-KO mice (n=11). Figure 1 shows representative tracings and concentrations response curves of PGE_2 and butaprost. Data are shown in Table 1.

The inhibition of mechanical activity can be due to a direct effect of on smooth muscle or, alternatively, by causing activation of nitrergic or purinergic neural pathways (Domenech *et al.,* 2011). Tissue incubation with TTX 1 μM, L-NNA 1 mM or MRS2500 1 μM did not significantly modify the inhibitory response elicited by neither PGE_2 (WT and EP_2-KO mice) nor butaprost (WT mice) (n=4 each) (Figure 1B). This finding suggests that both agonists are acting post-junctionally and the effect is not due to nerve-mediated inhibitory responses.

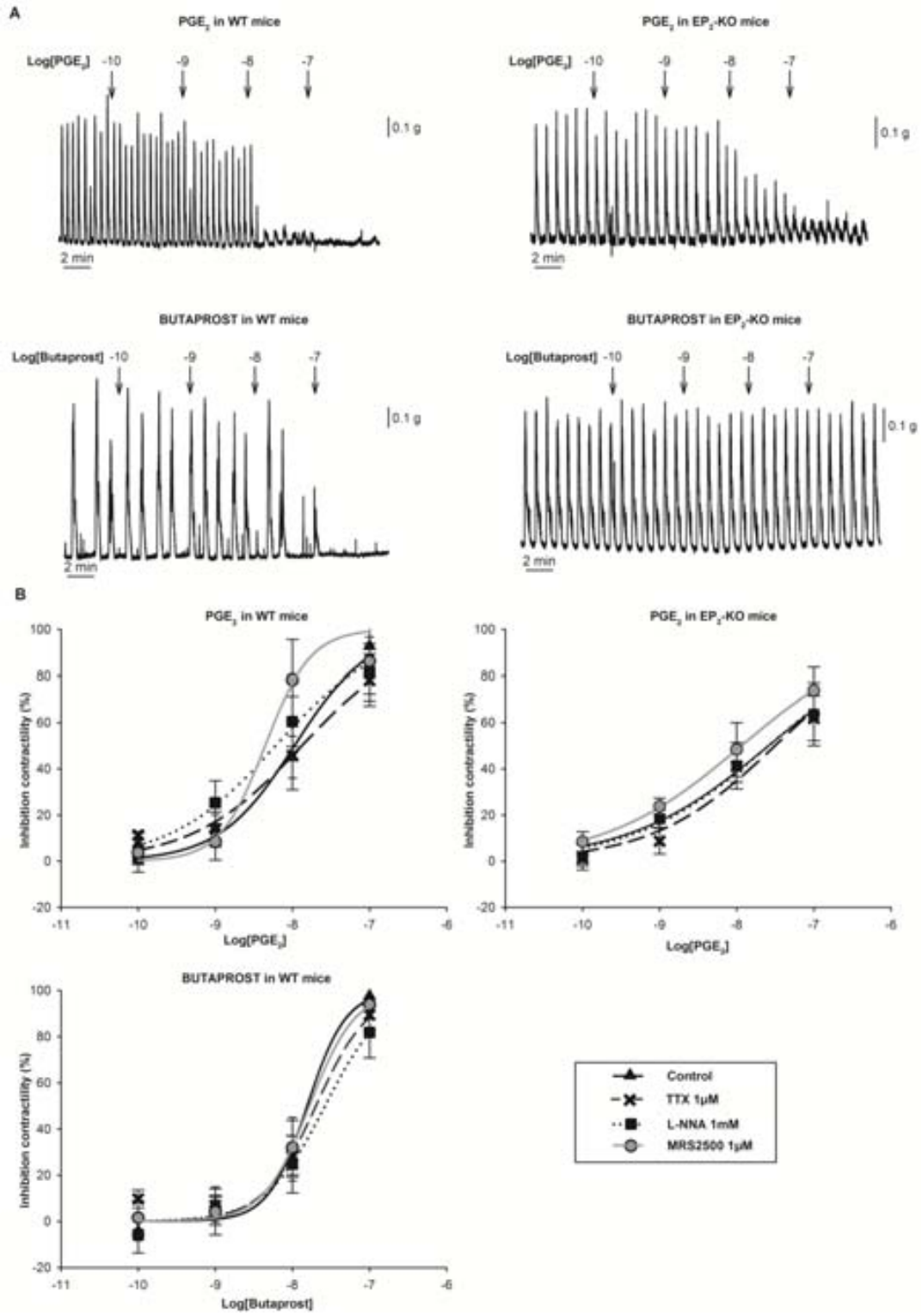

Figure 1. (A) Mechanical recordings showing the inhibition of spontaneous contractility of cumulative concentration-response curves (0.1, 1, 10, 100 nM) of PGE_2 and butaprost in WT (left) and EP_2-KO (right) mice. Notice that butaprost did not inhibit spontaneous contractions in EP_2-KO mice (see graph plot in Figure 2E). (B) PGE_2 (WT and EP_2-KO mice) and butaprost (WT mice) inhibited spontaneous contractility (calculated as the percentage of AUC) after incubation with TTX 1 µM, L-NNA 1 mM and MRS2500 1 µM. No statistically significant differences were observed (Two-Way ANOVA).

Table 1. Inhibitory effect of PGE_2 and butaprost in the presence of EP_2 (PF-04418948 0.1 µM) and EP_4 (L-161,982 10 µM) antagonists in WT and EP_2-KO mice.

	Protocol	WT mice			EP_2-KO mice		
		$LogEC_{50}$	**Hill slope**	**n**	**$LogEC_{50}$**	**Hill slope**	**n**
PGE_2 (0.1 nM-0.1 µM)	Control	-8.0±0.10	0.89±0.18	9	-7.6±0.11	0.77±0.14	8
	PF-04418948 0.1µM	-7.6±0.12	0.62±0.10	6	-7.6±0.14	1.07±0.29	5
	L-161,982 10µM	-7.7±0.13	0.92±0.22	4	No response		5
	PF-04418948 0.1µM + L-161,982 10µM	No response		4	-----		
Butaprost (0.1 nM-0.1 µM)	Control	-7.8±0.13	1.7±0.90	5	No response		11
	PF-04418948 0.1µM	No response		4	-----		

$LogEC_{50}$ and Hill slope values are represented as mean±S.E.M. The protocols which have not been performed are represented with a discontinuous line. No response: the agonist did not cause any effect in the presence of the corresponding antagonist(s). n is the number of different animals used in each protocol.

PGE_2 effect is mediated by EP_2 and EP_4 receptors: mechanical evidence.

In WT mice, tissue incubation with either the EP_2 receptor antagonist PF-04418948 0.1 µM (n=6) or the EP_4 antagonist L-161,982 10 µM (n=4) did not modify PGE_2 induced inhibitory responses (Figure 2A). However, incubation with both antagonists completely abolished the inhibitory effect exerted by PGE_2 (n=6, Figure 2A and C). These results demonstrate that both EP_2 and EP_4 receptors participate in PGE_2 inhibitory responses. Consistently, PGE_2 inhibition of spontaneous contractions was fully antagonized by L-161,982 10 µM in EP_2-KO mice (n=5) (Figure 2B). The effect of butaprost was fully antagonized by the EP_2 antagonist PF-04418948 0.1 µM in WT mice (n=4; Figure 2D and E). Data are shown in Table 1.

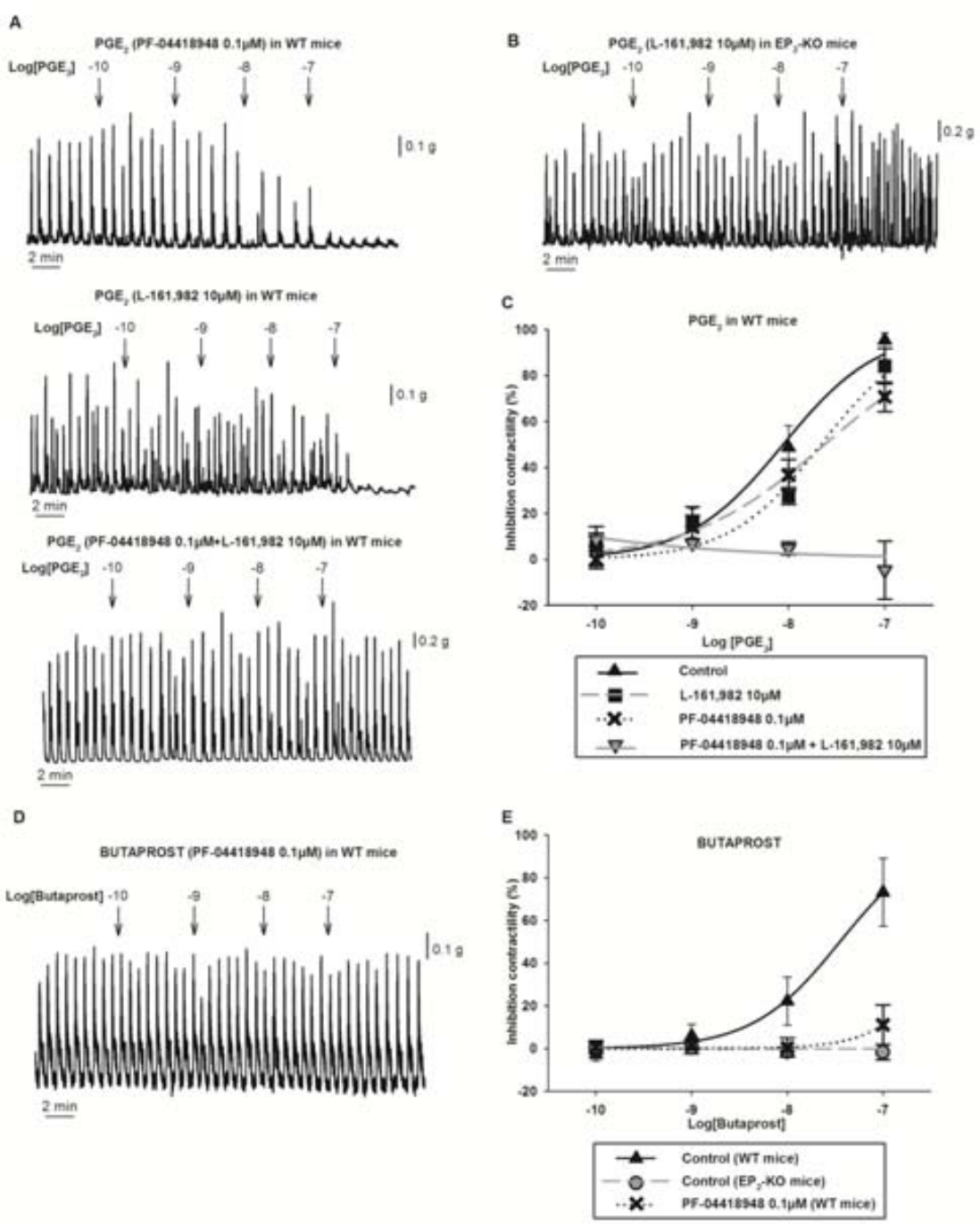

Figure 2. PGE_2 concentration dependently (0.1,1,10,100 nM) inhibited spontaneous contractions in WT mice after incubation with the EP_2 antagonist PF-04418948 0.1 μM (A top and C) and the EP_4 antagonist L-161,982 10 μM (A middle, and C). Notice that combination of both antagonists is needed to block PGE_2 inhibitory effect (A bottom and C). Incubation with L-161,982 10 μM antagonised PGE_2 effects in EP_2-KO mice (B). Butaprost (0.1,1,10,100 nM) did not inhibit spontaneous contractions after incubation with the EP_2 antagonist in WT mice and in EP_2-KO mice (D and E).

PGE_2 effect is mediated by EP_2 and EP_4 receptors: electrophysiological evidence

Tissue superfusion with PGE_2 1 µM caused a smooth muscle hyperpolarization in WT mice (-8.5±2.2 mV, n=4). Previous incubation with both EP_2 and EP_4 antagonists (PF-04418948 0.1 µM and L-161,982 10 µM) was needed to completely block the hyperpolarization induced by PGE_2 (Figure 3A and E). These results are consistent with a role for both receptors in PGE_2 mediated electrophysiological responses. In EP_2-KO mice, PGE_2-induced smooth muscle hyperpolarization (-8.2±2.6 mV, n=5) was similar to the one observed in WT animals (Figure 1B). As expected, PGE_2 hyperpolarization in EP_2-KO mice was not modified by the EP_2 antagonist (PF-04418948 0.1 µM) but was totally abolished with the subsequent tissue incubation with the EP_4 antagonist (L-161,982 10 µM). Butaprost 1 µM induced a smooth muscle hyperpolarization of -8.4±2.1 mV (n=5) in WT mice. In this case, butaprost-induced hyperpolarization was totally abolished with previous incubation with the EP_2 antagonist PF-04418948 0.1 µM (Figure 3C and G). Butaprost did not modify the RMP of SMCs in EP_2-KO mice (Figure 3D and G).

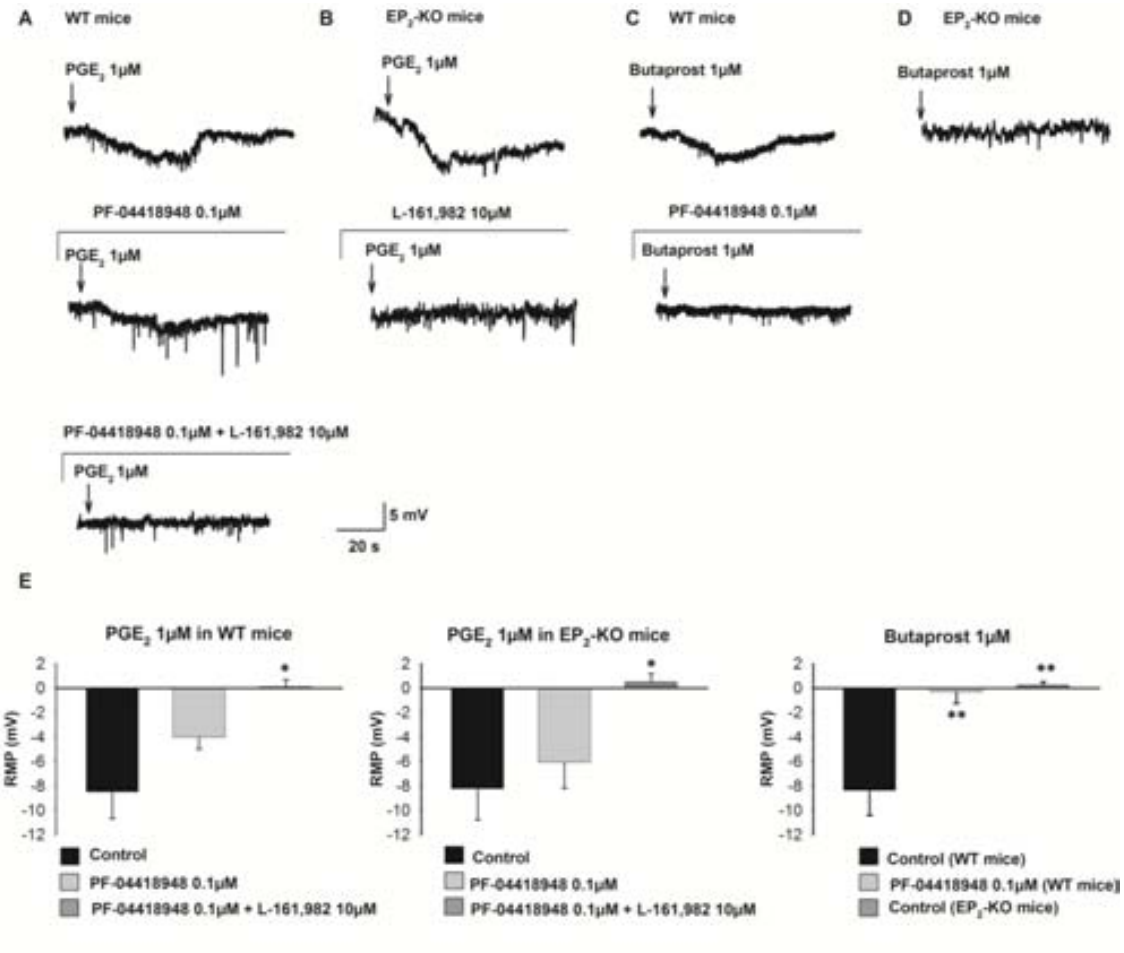

Figure 3. Electrophysiological tracings in WT and EP_2-KO mice showing the effects on colonic smooth muscle RMP of PGE_2 1 μM (A, B) and butaprost 1 μM (C, D) in control conditions (top) and after incubation with EP_2 and EP_4 antagonists (middle and bottom). (E) Histograms showing the effects of EP_2 and EP_4 antagonists on PGE_2 and butaprost induced hyperpolarization in WT and EP_2-KO mice. Both EP_2 and EP_4 antagonists are needed to block PGE_2 effects (WT mice). In contrast, L-161,982 10 μM inhibited PGE_2 mediated responses in EP_2-KO mice. PF-04418948 0.1 μM inhibited butaprost induced hyperpolarization. Notice the lack of effect of butaprost in EP_2-KO mice. One-Way ANOVA was performed followed by a Bonferroni's multiple comparison test (* $P<0.05$, ** $P<0.01$, compared to control).

Pharmacological characterization of EP_2 and EP_4 antagonists.

The effects of PGE_2 0.1 µM on spontaneous contractions were measured to characterise the antagonism elicited by PF-04418948 and L-161,982 on EP_2 and EP_4 receptors. As the combination of both antagonists was necessary to completely block PGE_2 responses, the inhibitory effect of each antagonist was studied in the presence of saturating concentrations of the other one. Accordingly, tissue was incubated with PF-04418948 0.1 µM to characterise the blockade of L-161,982 and tissue was incubated with L-161,982 10 µM to characterise the effect of PF-04418948. Under these experimental conditions, the EC_{50} values for L-161,982 and PF-04418948 were 5.1 nM and 10.6 nM respectively. In EP_2-KO mice, L-161,982 concentration-dependently inhibited PGE_2 responses (EC_{50}= 5.8 nM) (Figure 4 and Table 2).

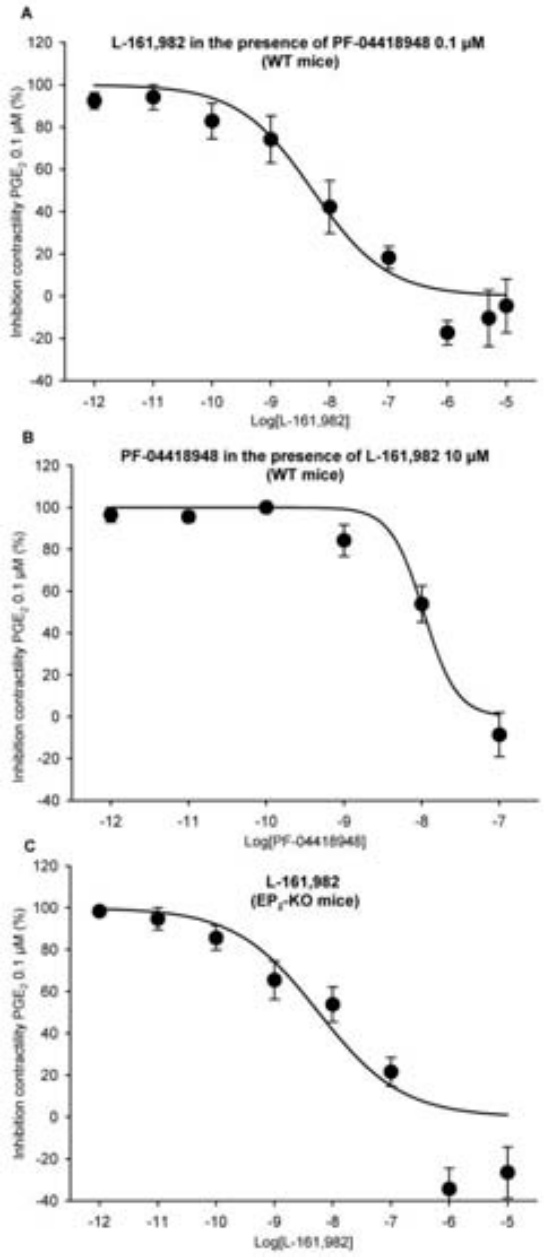

Figure 4. Plot graphs showing PGE_2 0.1 µM inhibition of spontaneous contractility after incubation with: (A) L-161,982 from 1 pM to 10 µM in the presence of PF-04418948 0.1 µM in WT mice; (B) PF-04418948 from 1 pM to 0.1 µM in the presence of L-161,982 10 µM in WT mice; and (C) L-161,982 from 1 pM to 10 µM in EP_2-KO mice.

Table 2. Inhibitory effect of L-161,982 and PF-04418948 on PGE_2 0.1 μM in WT and EP_2-KO mice.

	$LogEC_{50}$	**Hill slope**	**R^2**	**Degrees of freedom**
L-161,982 in the presence of PF-04418948 0.1 μM (WT mice)	-8.3±0.19	-0.69±0.18	0.84	32
PF-04418948 in the presence of L-161,982 10 μM (WT mice)	-8.0±0.07	-2.04±2.51	0.88	21
L-161,982 (EP_2-KO mice)	-8.2±0.22	-0.61±0.17	0.78	31

$LogEC_{50}$ and hill slope values are represented as mean±S.E.M. Each experimental value was obtained with a different preparation. Total number of animals: 7 WT and 5 EP_2-KO.

Neural mediated inhibitory responses were not modified by PGE_2.

As PGE_2 produced a cessation of spontaneous motility in the organ bath technique, we tested whether this effect was due to a potentiation of inhibitory neurotransmission. Purinergic neurotransmission was isolated using L-NNA 1 mM. Single pulses (0.3 ms) at increasing voltages of EFS (8-40 V) elicited MRS2500-sensitive IJPf, which increased their amplitude in a voltage-dependent manner. After a 15-min incubation with PGE_2 1 μM, the same protocol was performed in order to test its effect on purinergic neurotransmission (Figure 5A). No changes were observed in the amplitudes of the IJPf (Two-Way ANOVA n.s.) (Figure 5B). Nitrergic neurotransmission (IJPs) was tested in the presence of MRS2500 1 μM and with a 20-second train of 5 Hz uninterrupted EFS (Figure 5C). After a 15-min incubation with PGE_2, the amplitude of the IJPs was not modified (T test n.s.) (Figure 4D). EP_2-KO mice also proved to have intact purinergic (Figure 5E and G) and nitrergic (Figure 5F and H) neurotransmission (T test n.s.).

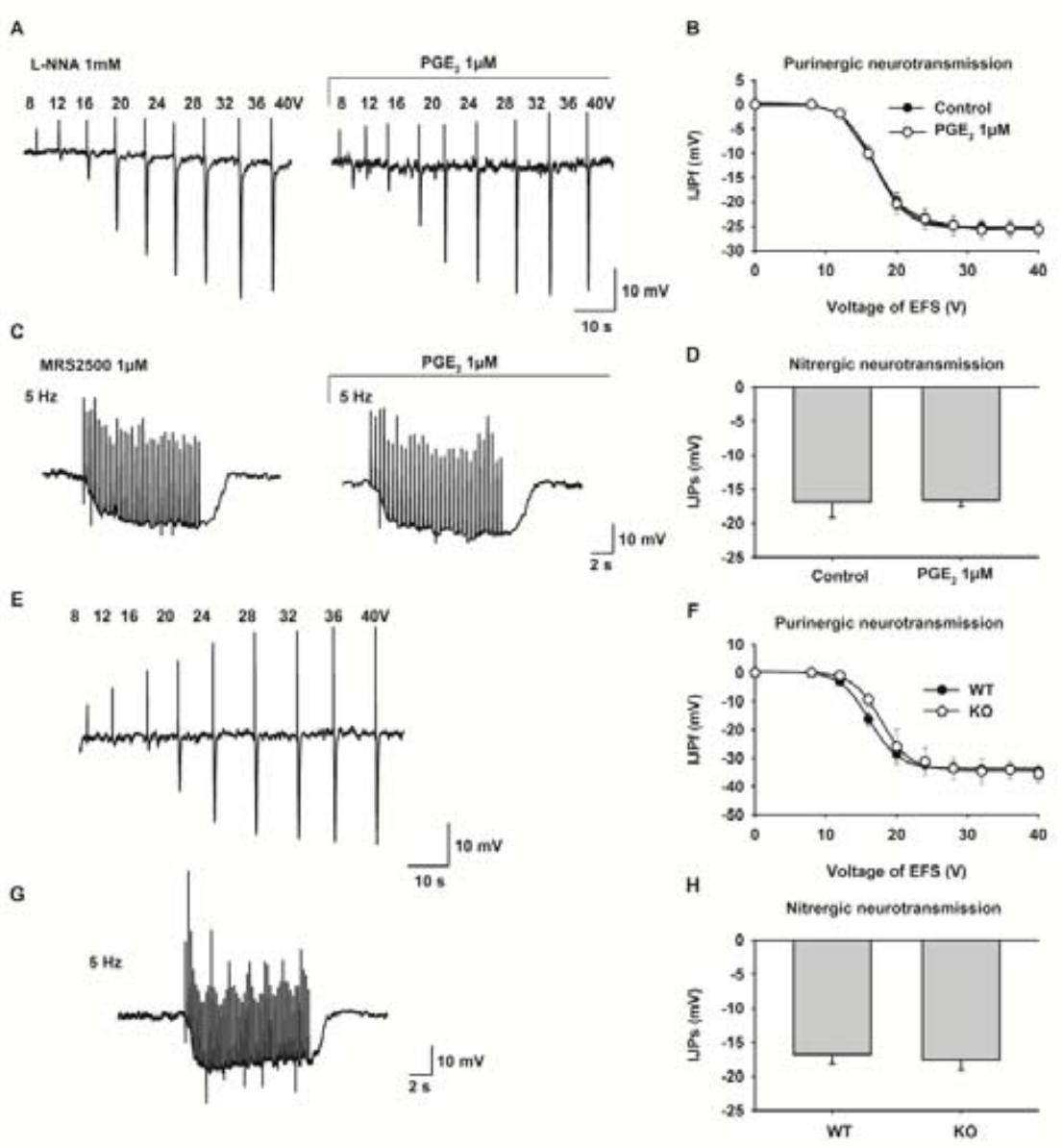

Figure 5. Electrophysiology recordings (A) and plot graph (B) showing the response elicited by a train of stimulation of increasing voltages in the presence of L-NNA 1 mM to study the purinergic component of the neurotransmission in control conditions and after PGE_2 incubation. Electrophysiology recordings (C) and histograms (D) represent the response elicited by continuous EFS (5 Hz, 20 s) in the presence of MRS2500 1 µM to study the nitrergic component of the neurotransmission in control conditions and after PGE_2 incubation. (E) and (F) represent the purinergic neurotransmission in EP_2-KO mice and the comparison between WT and EP_2-KO mice respectively. The nitrergic neurotransmission in EP_2-KO mice and the comparison between WT and EP_2-KO mice is represented in (G) and (H) respectively.

Both EP_2 and EP_4 receptors participate in the endogenous inhibitory tone

In order to assess the role of EP_2 and EP_4 receptors in the regulation of smooth muscle tone due to the endogenously produced PGE_2, we studied the effects of L-161,982 1 µM and PF-04418948 0.1 µM on spontaneous contractions and RMP. In mechanical experiments, L-161,982 1 µM was incubated during 15 min and afterwards PF-04418948 0.1 µM was added for 15 min more. The same protocol was also performed in the opposite order. In both cases, and in order to normalize data, 100 % was considered the initial AUC and the drug effect was calculated as a percentage of this initial responses. L-161,982 1 µM increased smooth muscle contractility to 198.3±13.8 % from initial AUC, and when PF-04418948 0.1 µM was added on top a further increase of spontaneous motility until 401.1±87.8 % was observed. Similar responses were obtained when the protocol was performed in the opposite order: the increase from basal AUC evoked by PF-04418948 0.1 µM was 279.7±81.1 % and posterior addition of L-161,982 1 µM further increased AUC to 517.6±131.5 % (One-Way ANOVA was performed followed by a Bonferroni's multiple comparison test, $P<0.05$, see figure 6). When the same protocol was performed with the microelectrode technique, the RMP depolarization induced by the two different combinations of the two antagonists was: 1- L-161,982 1 µM: 7.4±1.2 mV; +PF-04418948 0.1 µM: +9.2±1.4 mV, n=4; 2- PF-04418948 0.1 µM: 10.4±2.7 mV, +L-161,982 1 µM: 16.2±3.6 mV, n=6 (One-Way ANOVA was performed followed by a Bonferroni's multiple comparison test, $P<0.05$, see figure 6).

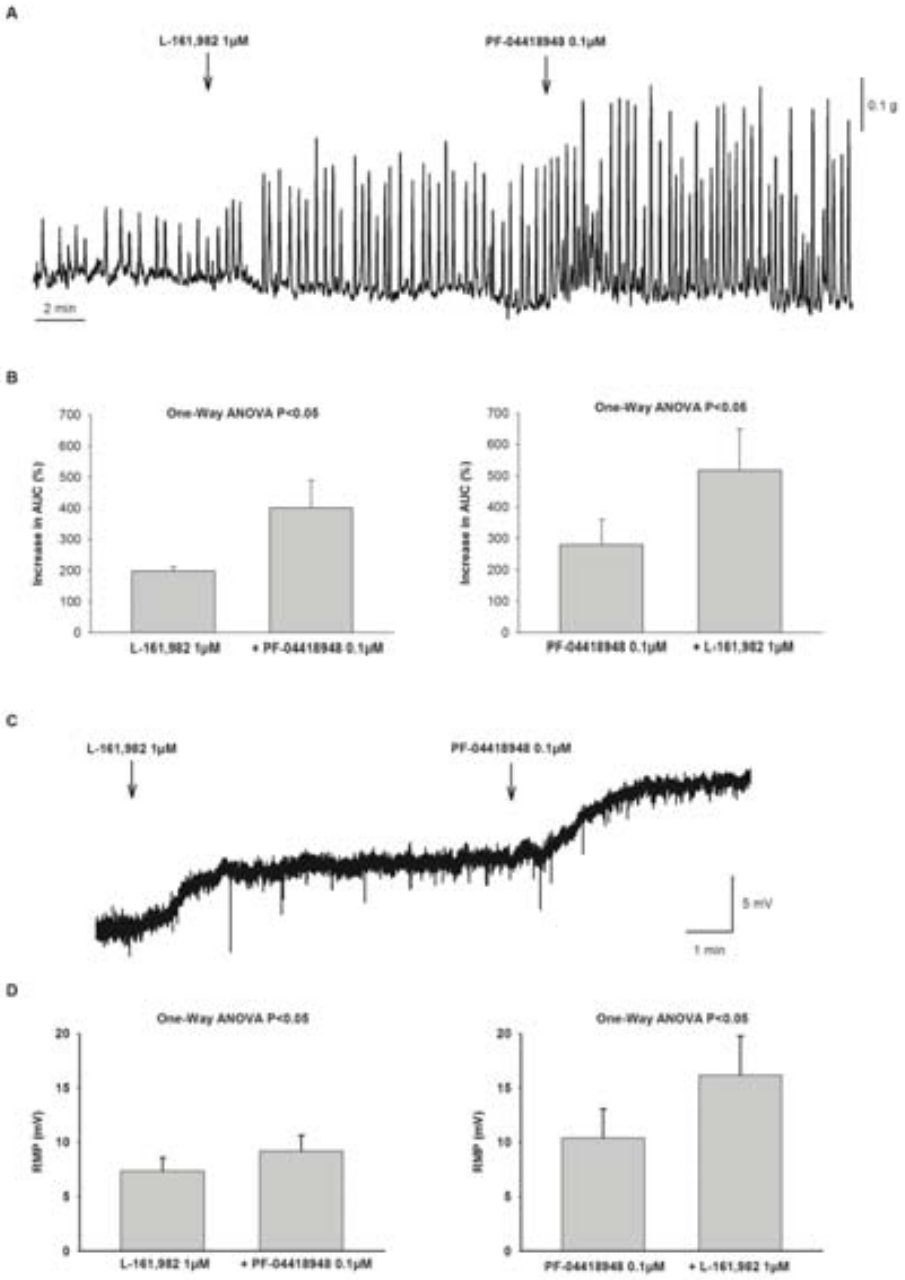

Figure 6. Increase in spontaneous contractility (A) and smooth muscle depolarization (C) caused by L-161,982 1 μM and the cumulative addition of PF-04418948 0.1 μM. Histograms showing the % of increase from basal AUC (B) and the depolarization (D) caused by the addition of EP_4 antagonist followed by EP_2 antagonist and vice versa. One-Way ANOVA was performed to compare the effects of EP_2 and EP_4 antagonists to basal RMP or basal AUC (100% in control).

DISCUSSION

In the present work we have characterized the inhibitory effects of PGE_2 on mouse colonic smooth muscle. Exogenous addition of PGE_2 caused smooth muscle hyperpolarization and cessation of spontaneous contractions in circularly oriented mice colonic strips. Our study demonstrates that both EP_2 and EP_4 post-junctional receptors mediate PGE_2 inhibitory effects. Moreover, we have reported that the blockade of EP_2 and EP_4 receptors increases spontaneous contractility, suggesting that endogenously produced PGs are acting on these receptors.

PGE_2 inhibits spontaneous motility through post-junctional receptors.

In several preparations of the GI tract, PGE_2 is able to cause both contraction and relaxation (Bennett *et al.*, 1968a;Bennett *et al.*, 1968b;Bennett *et al.*, 1981;Botella *et al.*, 1995;Dey *et al.*, 2006). Different effects are observed in different species, regions of the GI tract and muscle layers. This is probably due to different expression of EP receptors in different subclasses of enteric neurons, smooth muscle or ICC. Our results demonstrate that the main effect of PGE_2 in the mouse colonic circular layer is inhibitory, as it has been reported in the circular layer of the human colon (Bennett *et al.*, 1981).

Enteric inhibitory motor neurons have a crucial role in several GI physiological functions such as peristalsis or accommodation. Both in the human and rat colon, EFS induced IJP includes a purinergic IJPf ($P2Y_1$ mediated and MRS2500 sensitive) followed by a nitrergic IJPs (L-NNA sensitive) (Gallego *et al.*, 2011;Gallego *et al.*, 2008;Grasa *et al.*, 2009;Jimenez *et al.*, 2014). Taking into account that PGE_2 inhibits $P2Y_1$ mediated responses in human macrophages (Traves *et al.*, 2013), and that nitric oxide (NO) seems to be involved in the vascular relaxation induced by PGE_2 targeting EP_4 receptors (Hristovska *et al.*, 2007), the first aim of the present study was to elucidate the effects of PGE_2 both in purinergic and nitrergic components of the IJP. As previously described in the colon, single pulses of EFS elicit IJPf whereas high frequencies of stimulation result in an increase of the amplitude of the IJPs (Mane *et al.*, 2014). Our results show that both purinergic and nitrergic responses induced by EFS are present in tissue preparations from both WT and EP_2-KO mice. Neither nitrergic nor purinergic neurotransmission were substantially affected by PGE_2, suggesting that this molecule is not modifying neural mediated inhibitory pathways in the mouse colon. Indeed, inhibition of spontaneous contractility induced by both PGE_2 and butaprost persisted after incubation with the neural blocker TTX, the NO synthase inhibitor L-NNA or the $P2Y_1$ blocker MRS2500, suggesting a post-junctional effect. In the mouse and human colon, butaprost and PGE_2 induced responses were not blocked by the sodium channel blocker lidocaine, suggesting a

direct effect on smooth muscle (Fairbrother *et al.*, 2011). Thus, our results suggest that PGE_2 causes its inhibitory effect by acting directly on SMCs instead of inducing a pre-junctional release of inhibitory neurotransmitters (NTs). Consistent with this hypothesis, we have also demonstrated that both PGE_2 and butaprost cause smooth muscle hyperpolarization in mice colonic smooth muscle.

Both EP_2 and EP_4 receptors participate in the inhibitory responses induced by PGE_2.

Our results show that the inhibition of spontaneous contractions induced by PGE_2 is still produced in the presence of the EP_2 antagonist PF-04418948. Similarly, PGE_2 effect is also observed in the presence of the EP_4 antagonist L-161,982. The EC_{50} is similar in both cases suggesting that in this tissue PGE_2 is able to cause similar inhibitory responses both through EP_2 and EP_4 receptors. Therefore, a combination of both EP_2 and EP_4 antagonists is needed to block PGE_2 relaxant effect. Similar results have been obtained in our electrophysiological studies, since abolition of PGE_2 induced hyperpolarization requires the incubation with both antagonists. Moreover, in EP_2-KO mice, the EP_4 antagonist L-161,982 totally blocks PGE_2 induced hyperpolarization as well as inhibition of spontaneous contractility. Regarding the effects on mechanical activity, the calculated EC_{50} for PGE_2 in EP_2-KO mice is similar to the one obtained for PGE_2 in the presence of the EP_2 antagonist in WT animals. Moreover, butaprost is not able to cause smooth muscle hyperpolarization in EP_2-KO mice but induces a PF-04418948 sensitive hyperpolarization in WT animals. Similar results have been obtained in mechanical responses: the EC_{50} calculated for butaprost is similar to the EC_{50} obtained for PGE_2 in the presence of the EP_4 antagonist in WT animals. Taken together, all these results demonstrate that both EP_2 and EP_4 receptors participate in the inhibitory effect of PGE_2. Similar findings have been reported in other tissues such as human aortic SMCs, where histamine responses are attenuated by activation of both EP_2 and EP_4 receptors (Pantazaka *et al.*, 2013), and in mouse airway smooth muscle, where cholinergic contraction is also reduced by activation of both receptors (Srivastava *et al.*, 2013).

In the mouse colon, PF-04418948 and L-161,982 are selective EP_2 and EP_4 antagonists respectively.

Selective EP receptor antagonists are crucial tools to properly characterize PGE_2 responses. In this study, we have used two antagonists: L-161,982 10 µM, a selective EP_4 antagonist (Machwate *et al.*, 2001;Cherukuri *et al.*, 2007;Coskun *et al.*, 2013) and PF-04418948, a selective EP_2 antagonist (af Forselles *et al.*, 2011;Birrell & Nials, 2011). AH6809

has been previously used as an EP_2 receptor antagonist, since it has been able to block butaprost responses in the human and mouse colon and ileum (Fairbrother *et al.*, 2011). Nevertheless, AH6809 has similar affinity for EP_2, EP_3 and DP receptors (Abramovitz *et al.*, 2000). In contrast, PF-04418948 has shown a high selectivity for EP_2 receptors, being inactive on other EP receptors including EP_1, EP_3, EP_4 and DP receptors (af Forselles *et al.*, 2011). PF-04418948 inhibited PGE_2 mediated responses in cells expressing EP_2 receptors at the range of nM, demonstrating higher potency when compared to AH6809. Moreover, PF-04418948 evoked a rightward shift of the butaprost-mediated relaxant responses in human myometrium. To our knowledge, PF-04418948 has never been tested in GI tissues(af Forselles *et al.*, 2011). Our results demonstrate that: 1- PF-04418948 0.1 µM completely abolishes butaprost-induced colonic relaxation and hyperpolarization in WT mice; 2- 0.1 nM of PF-04418948 blocks the PGE_2 induced inhibition of mechanical activity after pre-incubation with L-161,982 10 µM; and 3- PF-04418948 does not modify neither electrical nor mechanical responses induced by PGE_2 in EP_2-KO mice. In order to compare the effects exerted through EP_2 and EP_4 antagonists, the blockade of PGE_2 induced responses of one antagonist was assessed in the presence of the another one (see table 2). Interestingly, the antagonism of L-161,982 on PGE_2 effect was almost identical in tissue preparations from WT animals previously incubated with PF-04418948 to that of EP_2-KO mice. These results indicate that PF-04418948 and L-161,982 act as selective and potent EP_2 and EP_4 antagonists respectively in the mouse colon and are potentially useful tools to properly characterise PGE_2 responses in GI tissues.

Endogenous activation of EP_2 and EP_4 receptors might be responsible for the endogenous inhibitory PG tone.

Constitutive and inducible PG synthesis has been demonstrated in colonic smooth muscle. Both COX-1 and COX-2 participate in PG production in both healthy and inflammatory states. COX-1 and COX-2 expression has been demonstrated in the muscular wall of human colon (Bernardini *et al.*, 2006). As COX-1 and COX-2 inhibitors enhance neural mediated cholinergic responses in the rat colon, it has been suggested that endogenously produced PGs modulate tissue contractility (Fornai *et al.*, 2006). Both COX-1 and COX-2 are expressed in murine proximal colon and both Indomethacin and GR253035X (COX-2 inhibitor) increase spontaneous contractions (Porcher *et al.*, 2004). All these results suggest that endogenous production of PGs, probably including PGE_2, might maintain the colonic SMCs hyperpolarised and inhibited. In the same line with this hypothesis, we have demonstrated that pre-incubation with PF-04418948 0.1 µM (EP_2 receptor antagonist) and L-161,982 1 µM (EP_4

receptor antagonist) results in smooth muscle depolarization and increase of the contractile activity. The additive effect of both antagonists suggests that both EP_2 and EP_4 receptors are involved in the response. Taken together, these findings suggest that endogenous production of PGs, possibly including PGE_2, may be exerting an inhibitory control of smooth muscle excitability by acting on both EP_2 and EP_4 relaxant receptors. In inflammatory processes of the gut, PGE_2 and EP receptors participate in the impaired colonic transit (Takafuji *et al.*, 2000;Cong *et al.*, 2007;Lin *et al.*, 2012). Consequently, if the ongoing production of PGs is increased during inflammation, COX-2 inhibitors or alternatively EP antagonists are potential pharmacological targets to treat motility disorders.

In the present work we have shown that both EP_2 and EP_4 receptors participate in PGE_2 mediated smooth muscle hyperpolarization and inhibition of contractility in mouse colon. As far as we know, it is the first time that a selective antagonist of EP_2 receptor has been tested in the GI tract. Our results are consistent with the presence of a constitutive production of PGs acting on both EP_2 and EP_4 receptors. As an enhanced PG synthesis or an altered EP receptor expression can contribute to the dismotility observed during gut inflammation, the present study can be considered a first step to investigate, in future studies, the involvement of EP_2 and EP_4 receptors in GI motility disorders.

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CHAPTER 3

Potential role of the gaseous mediator Hydrogen Sulphide in inhibiting human colonic contractility

ABSTRACT

Hydrogen sulphide (H_2S) is an endogenous signalling molecule that might play a physiologically relevant role in gastrointestinal motility. Cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) are two enzymes responsible for H_2S production. D,L-propargylglycine (PAG) is a CSE inhibitor whereas Aminooxyacetic acid (AOAA) is a CBS inhibitor. The characterization of H_2S responses and its mechanism of action is crucial to define H_2S function. Human colonic strips were used to investigate the role of H_2S on contractility (muscle bath) and smooth muscle electrophysiology (microelectrodes). Sodium hydrogen sulphide (NaHS) was used as a H_2S donor. Combination of PAG and AOAA depolarized (5-6 mV, n=4) the smooth muscle and elicited a transient increase in tone (260.5±92.8 mg, n=12). No effect was observed on neural mediated inhibitory junction potential or relaxation. In the presence of tetrodotoxin 1 µM, NaHS concentration-dependently inhibited spontaneous contractions (EC_{50}=329.2 µM, n=18). This effect was partially reduced by the Guanylyl cyclase inhibitor ODQ 10 µM (EC_{50}= 2.6 µM, n=12) and by L-NNA 1 mM (EC_{50}=1.4 mM, n=8). NaHS reversibly blocked neural mediated cholinergic (EC_{50}=2 mM) and tachykinergic (EC_{50}=5.7 mM) contractions. NaHS concentration dependently reduced the increase in spontaneous mechanical activity (area under curve) induced by carbachol (EC_{50}=1.9 mM) and neurokinin A (EC_{50}=1.7 mM). H_2S might be an endogenous gasomediator regulating human colonic contractility. Its inhibitory effect could be mediated by a direct effect on smooth muscle with a possible synergistic effect with nitric oxide, as well as by an interaction with the cholinergic and tachykinergic neural mediated pathways.

INTRODUCTION

Hydrogen sulphide (H_2S) is nowadays recognized as the third gasotransmitter along with nitric oxide (NO) and carbon monoxide (CO). H_2S fulfils, at least in part, the criteria to be considered a gasotransmitter with relevant functions in the gastrointestinal (GI) tract (Linden *et al.*, 2010;Jimenez, 2010).

H_2S is synthesized mainly by two pyridoxal phosphate dependent enzymes cystathionine β-synthase (CBS; EC 4.2.1.22) and cystathionine γ-lyase (CSE; EC 4.4.1.1) that use L-cysteine as substrate (Stipanuk & Beck, 1982). In addition, a third non-pyridoxal-phosphate-dependent enzyme, 3-mercaptopyruvate sulfurtransferase (3MST; EC 2.8.1.2), has been proposed as a H_2S generating enzyme in combination with cysteine aminotransferase (EC 2.6.1.3) (Stipanuk & Beck, 1982;Shibuya *et al.*, 2009a;Shibuya *et al.*, 2009b). Due to bacterial production, H_2S is present in the lumen of the human large intestine at high concentrations (range of milimolar). However, the amount of H_2S that reaches the subepithelial space is very low due to the capability of fecal components to bind the sulphide as well as to colonic epithelial cells metabolic barrier (Wallace *et al.*, 2012). This H_2S source could exert significant effects when the epithelial barrier is dysfunctional or damaged (Wang, 2002;Schicho *et al.*, 2006;Wallace *et al.*, 2012).

H_2S endogenous production has been demonstrated in the GI tract and both CBS and CSE have been localized along the gut (Linden *et al.*, 2010;Martin *et al.*, 2010;Gil *et al.*, 2011). CBS and CSE have been detected in enteric neurons, interstitial cells of Cajal (ICCs) (Schicho *et al.*, 2006) and smooth muscle cells (SMCs) (Hennig & Diener, 2009;Gil *et al.*, 2011); therefore it is possible that different cell types are able to produce H_2S.

Studies performed with the H_2S donor NaHS have demonstrated that H_2S has pro-secretory effects in several species including the human and rat colon (Schicho *et al.*, 2006;Hennig & Diener, 2009). NaHS presynaptically potenciates fast excitatory cholinergic nicotinic post-synaptic potentials (fEPSPs) in splanchnic nerves which might contribute to the inhibition of colonic motility in mice (Sha *et al.*, 2013). NaHS also causes concentration-dependent relaxation of smooth muscle contractility and inhibition of peristalsis (Hosoki *et al.*, 1997;Teague *et al.*, 2002;Gallego *et al.*, 2008a;Dhaese *et al.*, 2010;Gil *et al.*, 2011), and might interact directly with SMCs causing hyperpolarization and therefore smooth muscle relaxation (Gallego *et al.*, 2008a). Studies performed with laboratory animals have demonstrated that NaHS is able to interact with the cholinergic pathway. Recently, Gil et al., demonstrated a reduction of cholinergic excitatory junction potential (EJP) induced by NaHS in rat colon (Gil *et*

al., 2013). In the respiratory system, H_2S reversibly inhibited acetylcholine (ACh)-induced calcium oscillations responsible for the contraction of airway SMCs (Castro-Piedras & Perez-Zoghbi, 2013). Similar findings have been reported in guinea-pig ileum and rat jejunum, where H_2S significantly reduced cholinergic mediated contractions (Teague *et al.*, 2002;Kasparek *et al.*, 2012).

Scarce data is available about the possible role of endogenous H_2S in the GI tract. A role for endogenous H_2S in smooth muscle inhibitory tone has been recently proposed, as both CBS and CSE inhibitors were able to depolarize resting membrane potential (RMP) and to increase spontaneous motility in the rat colon (Gil *et al.*, 2011). Endogenous H_2S might also participate in the CO mediated transwall gradient of the RMP in the mouse colon (Sha *et al.*, 2014).

The vast majority of the papers refer to the potential role of H_2S in animal models. However, little is known about the potential role of H_2S in the human GI tract (Schicho *et al.*, 2006;Gallego *et al.*, 2008a). Accordingly, in the present work we have investigated the potential role of H_2S in human colonic excitability and contractility using inhibitors of CSE and CBS. Moreover, we have investigated the inhibitory effect of NaHS on spontaneous and neural mediated contractions.

MATERIALS AND METHODS

Human tissue preparation

Tissue specimens of human sigmoid colon were obtained from patients undergoing colon resections for neoplasm. Colon segments from macroscopic marginal regions were collected and transported in cold saline buffer. Tissue was placed in Krebs solution on a dissection dish and the mucosal layer was carefully removed. Muscle strips (10x4 mm) were cut oriented in the circular direction. Patients provided informed consent and all the experimental procedures were approved by the ethics committee of the Hospital of Mataró (Barcelona, Spain) CEIC code 04/09.

Intracellular microelectrode recordings

Muscle strips were pinned to the base of a Sylgard coated chamber and continuously perfused with Krebs solution. Strips were allowed to equilibrate for approximately 1 hour before recording. Circular SMCs were impaled with sharp glass microelectrodes filled with 3 M KCl (30-60 MΩ). Membrane potential was measured using standard electrometer Duo773 (WPI Inc., Sarasota, FL, USA). Tracings were displayed on an oscilloscope 4026 (Racal-Dana Ltd., Windsor, UK) and simultaneously digitalized (100 Hz) using PowerLab 4/30 system and Chart 5 software for Windows (all from ADInstruments, Castle Hill, NSW, Australia). Nifedipine (1 μM) was used to abolish the mechanical activity and obtain stable impalements. Electrical field stimulation (EFS) was applied using two silver chloride plates placed perpendicular to the longitudinal axis of the preparation and 1.5 cm apart. Train stimulation had the following parameters: total duration, 100 ms; frequency, 30 Hz; pulse duration, 0.3 ms and increasing amplitude strengths of 5, 10, 12, 15, 17, 20, 25, 30 and 50 V. RMP was measured before and after drug addition. The amplitude of the inhibitory junction potential (IJP) was measured in control conditions and after infusion of the drugs.

Mechanical studies

Mechanical activity was studied in a 10 mL organ bath. Circularly-oriented preparations were tied to an isometric force transducer (Harvard VF-1 Harvard Apparatus Inc., Holliston, MA, USA) using 2/0 silk thread. The isometric force transducer was connected to an amplifier to record the mechanical activity. Data were digitalized (25 Hz) using Datawin1 software (Panlab-Barcelona, Spain) coupled to an ISC-16 A/D card installed in a PC computer. A

tension of 4 g was applied to the tissue and was allowed to equilibrate for 1 hour. After this period, strips displayed spontaneous phasic activity.

EFS was applied through two platinum electrodes placed on the support holding the tissue. Different parameters of EFS and different pharmacological conditions were used to reveal inhibitory and excitatory neural mediated responses. To study the inhibitory neuromuscular transmission, preparations were studied in classical "non-adrenergic, non-cholinergic conditions" (NANC). In this case, Krebs containing atropine, propranolol and phentolamine (all at 1 μM) was used and EFS was applied for 2 minutes (pulse duration 0.4 ms, frequency 2 Hz and amplitude 50 V) in order to inhibit spontaneous myogenic contractions. In contrast, in order to study the excitatory neuromuscular transmission, Krebs solution contained phentolamine and propranolol (both at 1 μM) and experiments were performed in "non-nitrergic, non-purinergic" conditions. In this case, tissue was incubated with L-NNA (1 mM) and MRS2500 (1 μM) to avoid both nitrergic and purinergic neural mediated inhibitory responses (Gallego *et al.*, 2008b;Grasa *et al.*, 2009;Gallego *et al.*, 2011;Jimenez *et al.*, 2014). To study the cholinergic component, EFS was applied for 1 second (pulse duration 0.4 ms, frequency 50 Hz, amplitude 50 V). To study the tachykinergic component, "non-nitrergic, non-purinergic" conditions were also used and atropine (10 μM) was added to avoid neural mediated cholinergic responses. In this case EFS was applied for 10 seconds (pulse duration 0.4 ms, frequency 50 Hz, amplitude 50 V).

Cumulative concentration-response curves of NaHS (10, 30, 60, 100, 300, 600, 1000, 2000 and 3000 μM) were performed in the presence of the neural blocker tetrodotoxin (TTX) at 1 μM and after incubation with ODQ (10 μM) and L-NNA (1 mM). Cumulative concentration-response curves of HA (0.01, 0.1, 1, 10 and 100 μM) were performed in control conditions and in the presence of TTX (1 μM) and the guanylyl cyclase (GC) inhibitor ODQ (10 μM). Carbachol-induced responses were studied by means of a cumulative concentration-response curve (0.01, 0.1, 1, 3 and 10 μM) in control conditions and after incubating with NaHS or atropine both in normal and in calcium-free Krebs solution. Neurokinin A (NKA) effects were also studied by performing a cumulative concentration response curve (0.1, 1, 10 and 100 nM) in control conditions and after incubation with NaHS or with GR159897 (NK_2 receptor antagonist).

Solutions and drugs

The composition of the Krebs solution was (in mM): glucose, 10.1; NaCl, 115.5; $NaHCO_3$, 21.9; KCl, 4.6; NaH_2PO_4, 1.1; $CaCl_2$, 2.5 and $MgSO_4$, 1.2 (pH 7.3-7.4). The Krebs solution (37±1ºC) was bubbled with carbogen (95% O_2 and 5% CO_2).

The following drugs were used: aminooxyacetic acid (AOAA), D,L-propargylglycine (PAG), hydroxylamine (HA), carbachol, nifedipine, N^{ω}-nitro-L-arginine (L-NNA), phentolamine, 1-H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ), nifedipine, atropine sulphate, sodium hydrogen sulphide (NaHS), neur okinin A (NKA) (Sigma Chemicals, St. Louis, USA), 5-Fluoro-3-[2-[4-methoxy-4-[[(R)-phenylsulphinyl]methyl]-1-piperidinyl]ethyl]-1H-indole (GR159897) (Santa Cruz Biotechnology, San Diego, CA, USA), propranolol, (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) (Tocris, Bristol, UK), tetrodotoxin (TTX) (Latoxan, Valence, France). Stock solutions were made by dissolving drugs in distilled water except for: nifedipine and ODQ which were dissolved in 96% ethanol, GR159897 dissolved in DMSO, L-NNA dissolved in Krebs by sonication and AOAA dissolved in Krebs and the pH was adjusted to 7.4 by using NaOH.

Data analysis and statistics

Cumulative concentration-response curves of H_2S using NaHS as a donor were performed in order to calculate the EC_{50}. To normalize data, the percentage of inhibition of the drugs was calculated by considering the area under curve (AUC) before the addition of the H_2S donor as 100%. The effect of the drugs on NaHS concentration-response curve was assessed comparing different strips from the same patient. The differences between groups were compared by Two-way ANOVA. EC_{50} were calculated using conventional sigmoid dose-response curve with variable slope.

Cumulative concentration-response curves of carbachol and NKA were performed in control conditions, after incubation with NaHS and after incubation with muscarinic and NK_2 antagonists (atropine and GR159897 respectively). To study the effects on tone, increase from initial baseline (in grams) was calculated. To normalize data, the value obtained for the tone increase and AUC at the maximum dose of the agonist (carbachol 10 μM and NKA 0.1 μM) in control conditions was considered 100%. Different protocols with different antagonists were performed in different strips, but the control cumulative concentration-response curve of the corresponding agonist was performed at the beginning of the protocol each time.

One-way ANOVA was used to study the effects of NaHS, atropine or GR159897 on the contractile response induced by EFS. Two-way ANOVA was used: 1- to evaluate the effect of different concentrations of NaHS on spontaneous contractions before and after drug addition, 2- to evaluate the effects of concentration-response additive carbachol curves in control conditions and after incubating with atropine or NaHS and 3- to evaluate the effects of cumulative concentration-response NKA curves in control conditions and after incubating with GR159897 or NaHS. Concentration-response curves were calculated using non-linear regression and EC_{50} was estimated in the absence and presence of the antagonist (when the effect was expressed as percentage of maximum response, constraints were fixed at 0 (bottom) and 100 (top)).

Differences in the RMP before and after infusion of different drugs were compared by Paired T-test. The differences between the amplitude of the IJP before and after drug infusion were compared by Two-way ANOVA.

Data are expressed as mean ± S.E.M. and statistical significance was considered when $P<0.05$. "n" values indicate the number of samples. Statistical analysis and curve fit were performed with GraphPad Prism 6.00, GraphPad Software, San Diego, California, USA.

RESULTS

Effect of H_2S synthesis inhibitors on smooth muscle RMP and spontaneous contractions.

Human colonic strips circularly oriented displayed spontaneous phasic contractions at a frequency of about 3 cycles per minute. Tissue incubation with both the CSE inhibitor PAG (2 mM) and the CBS inhibitor AOAA (1 mM) caused a mild (260.5±92.8 mg) and transient (about 5 min) increase in tone (Figure 1A), but did not modify spontaneous contractility (AUC in control conditions: 35.2 ± 5.6 g min^{-1} vs. PAG 2 mM+AOAA 1 mM: 35.4 ± 6.0 g min^{-1}; n = 12; T test n.s.). No effect was observed when each inhibitor was administered alone (n=11; data not shown). A combination of a PAG 2 mM and AOAA 1 mM depolarized 5-6 mV the RMP of the SMCs (control RMP: -38.9 ± 1.8 mV vs. PAG 2 mM+AOAA 1 mM: -33.3 ± 2.0 mV; n= 3; T test $P<0.05$; Figure 1A). HA, a frequently used CBS inhibitor, reduced spontaneous contractions in a concentration-dependent manner both in control conditions (EC_{50}= 2.8 µM; n=5) and in the presence of the neural blocker TTX at 1 µM (EC_{50}= 2.0 µM, n=6; Figure 1B). The GC inhibitor ODQ (10 µM) shifted the control cumulative concentration-response curve of HA to the right (EC_{50}= 30.5 µM, n=5; Two-Way ANOVA $P<0.001$; Figure 1B).

Effect of PAG and AOAA on inhibitory neuromuscular transmission.

The effects of AOAA and PAG were tested on IJP and relaxation induced by EFS. Consistent with the increase in driving forces due to smooth muscle depolarization, combination of AOAA (1 mM) and PAG (2 mM) slightly increased the amplitude of the IJP (n = 4; Two-Way ANOVA $P<0.001$; Figure 1C and D). No effect was observed on EFS-induced inhibition of myogenic spontaneous contractility (Control: 98.2±1.0% vs. PAG 2 mM+AOAA 1 mM: 92.4±7.6; n = 4; ns) (Figure 1C).

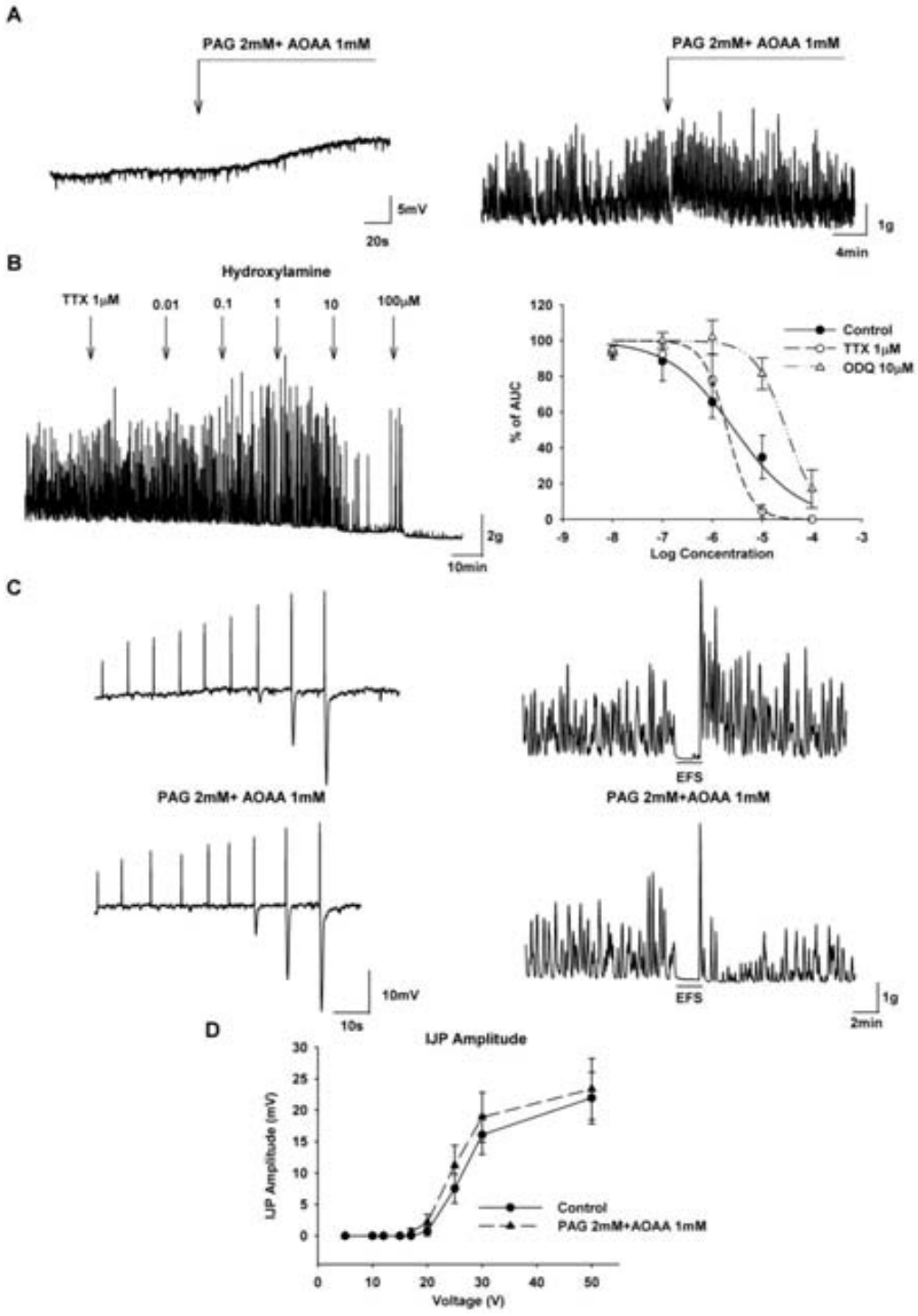

Figure 1. (A) Intracellular microelectrode (left) and mechanical recordings (right) showing the depolarization of smooth muscle RMP and the transient increase in tone elicited by a combination of PAG (2 mM) and AOAA (1 mM). (B) Mechanical recording showing the inhibitory effect of cumulative concentrations of HA (from 0.01 to 100 μM) (left) and graphical plot representing the concentration response-curves of HA in control conditions and in the presence of the neural blocker TTX (1 μM) and the GC inhibitor ODQ (10 μM) (right). Notice the rightward shift of the curve in the presence of ODQ (Two-Way ANOVA $P<0.001$). (C) Intracellular microelectrode recording (left) and mechanical recording (right) showing the effect of PAG (2 mM) and AOAA (1 mM) on the IJP and EFS-induced relaxation. (D) Graphical plot showing the slight increase on the IJP amplitude elicited by PAG and AOAA (Two-Way ANOVA $P<0.001$).

Effect of NaHS on myogenic spontaneous contractions

As previously described (Gallego *et al.*, 2008a), the H_2S donor NaHS concentration-dependently inhibited spontaneous contractions in the presence of TTX (1 µM) (EC_{50}=329.2 µM; log EC_{50}=-3.48±0.06; n=18; Figure 2). Previous data suggest that H_2S might interact with the nitrergic pathway, and some of its effects could be mediated via activation of GC. Tissue incubation with either ODQ (10 µM) or L-NNA (1 mM) (both in the presence of TTX) reduced the inhibitory effect of NaHS (EC_{50}=2.6 mM and EC_{50}= 1.4 mM respectively). See Figure 2 and Table 1.

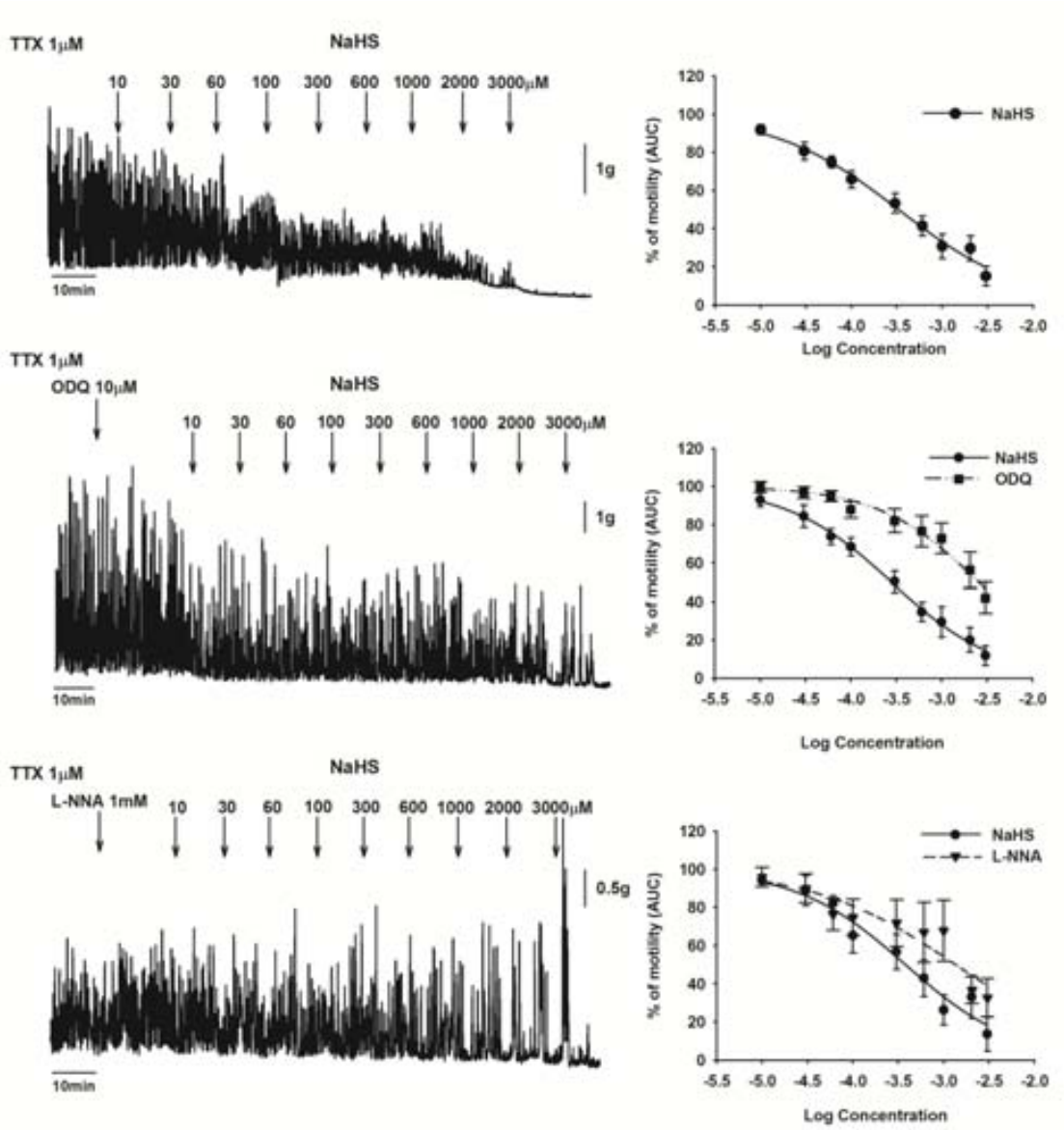

Figure 2. Mechanical recordings (left) and graphical plots (right) showing (from top to bottom) the inhibitory effect of cumulative concentration-response curves of NaHS (from 10 to 3000 µM) in human colonic strips in the presence of TTX (1 µM) and the partial reduction of NaHS effects by previous incubation with ODQ (10 µM) or L-NNA (1 mM). See Table 1.

Table 1. Log EC_{50} calculated from concentration-response cumulative curves, comparison was performed between smooth muscle strips from the same patient.

Drug	NaHS (Vehicle)	NaHS (Drug pre-treatment)	N	P
ODQ (10 µM)	Log EC_{50}=-3.14±0.05	Log EC_{50}=-2.59±0.09	12	<0.0001
L-NNA (1 mM)	Log EC_{50}=-3.42±0.09	Log EC_{50}=-2.87±0.16	8	0.003

EFS-induced cholinergic contraction is inhibited by previous incubation with NaHS.

Since several excitatory neurotransmitters (NTs) might participate in nerve mediated contractile responses, we designed a protocol of EFS to mainly produce neural mediated atropine sensitive cholinergic responses. First, "non-nitrergic, non-purinergic" conditions (tissue incubation with L-NNA (1 mM) and MRS2500 (1 µM)) were used to avoid possible nitrergic and purinergic inhibitory responses as well as the corresponding rebound contraction that might contribute to the nerve mediated responses (Auli *et al.*, 2008;Gallego *et al.*, 2011). Under these pharmacological conditions, EFS (50 V, 50 Hz, 0.4 ms pulse duration, 1 s train duration) elicited an atropine-sensitive contractile response (EC_{50}= 0.05 µM, $LogEC_{50}$= -7.30±0.13, n=3) that was almost abolished with 1 µM of atropine (Figure 3A and B). Accordingly, the mechanical response to EFS is mainly cholinergic under these experimental conditions. Additive concentrations of NaHS (15 minutes) were used to investigate whether H_2S was able to reduce neural mediated cholinergic responses. NaHS concentration-dependently inhibited EFS-induced contractions (EC_{50}= 2 mM, $LogEC_{50}$= -2.7±0.27, n=9). The concentrations of NaHS causing a significant reduction of the nerve mediated cholinergic response were 3 mM and 10 mM, being the amplitude of the control contraction (15.9±2.9 g) reduced to 5.4±2.7 g and 3.3±2.5 g respectively (Figure 3C and D). The amplitude of the cholinergic EFS-induced contraction recovered to 84.1±18.8 % of the original response (tested in 3 different strips from different patients) after 30 min washout, demonstrating the viability of the tissue and the recovery of the original response after NaHS exposure.

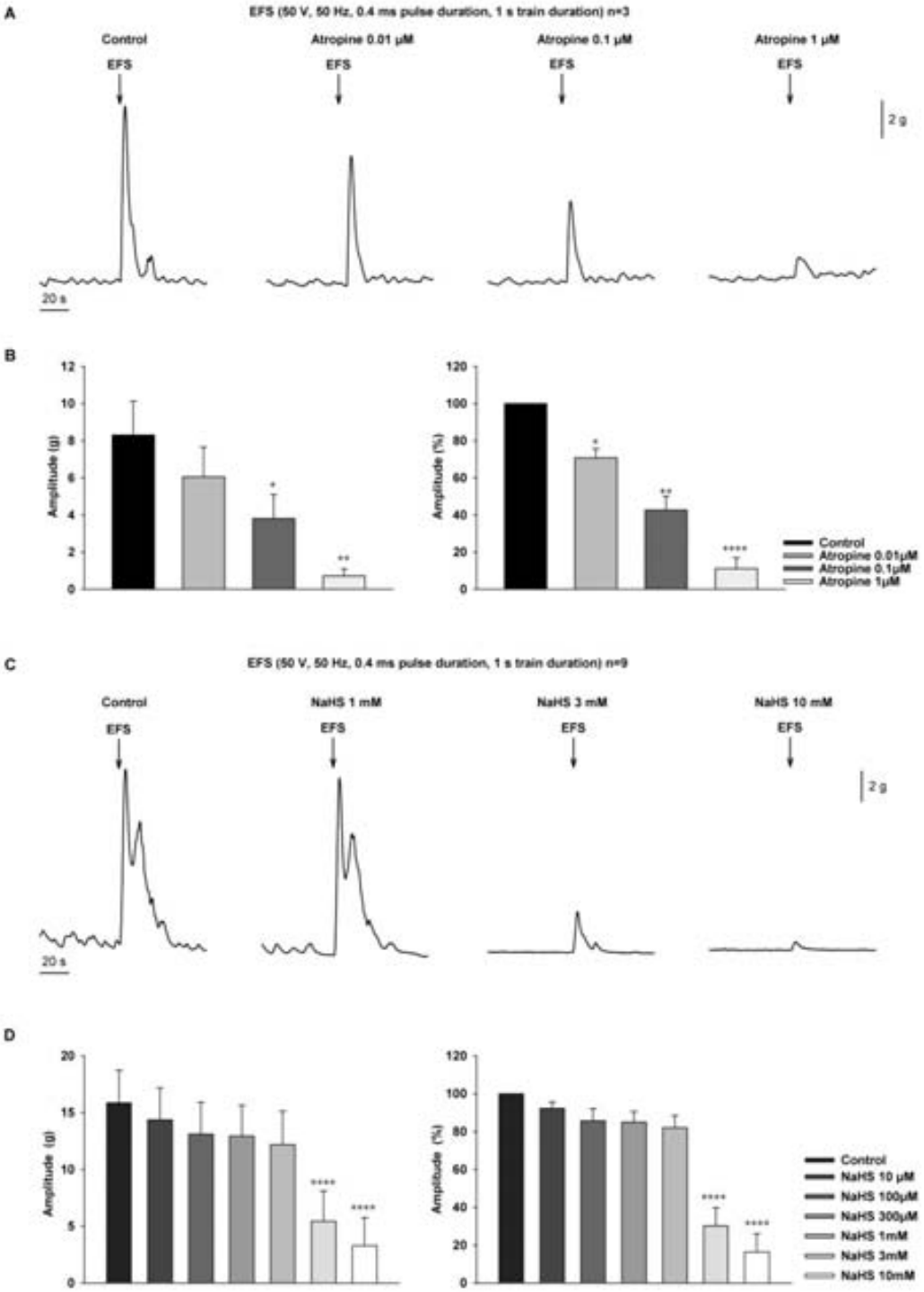

Figure 3. (A) Mechanical recordings showing the contraction elicited by EFS (50 V, 50 Hz, 0.4 ms, 1 s train duration) in control conditions and after incubation with atropine (0.01, 0.1 and 1 µM). (B) Histograms showing the reduction of the amplitude of EFS-induced contraction (raw data left and in percentage of the original response right) by additive concentrations of atropine (0.01 µM to 1 µM). (C) Tracings showing the contraction elicited by EFS in control conditions and after incubation with NaHS (1, 3 and 10 mM). (D) Histograms (raw data left and percentage of the control response right) showing the inhibition of the contractile response after incubation with additive concentrations of NaHS (from 10 µM to 10 mM). One-way ANOVA was performed followed by a Bonferroni's multiple comparison test (*<0.05, **<0.01, ***<0.001, **** P<0.0001).

Carbachol-induced responses are inhibited by NaHS.

Cumulative concentration-response curves of carbachol (from 0.01 to 10 µM) caused a progressive increase in the contractile response superimposed with spontaneous myogenic contractions. Two parameters were measured: tone (quantification of the increase in the baseline) and AUC (quantification of the total response including spontaneous contractions). Carbachol concentration dependently increased both the AUC and the tone of the preparation, reaching a maximum at 10 µM of carbachol of 471.82±50.22 g min^{-1} and 7.83±1.12 g respectively (n=12). Carbachol-induced responses were abolished by atropine (1 µM) (n=8). Previous incubation with NaHS (0.3 to 3 mM) reduced carbachol contractile responses in a concentration-dependent manner, being significantly reduced after NaHS 3 mM (increase in tone induced by carbachol 10 µM in the presence of NaHS 3 mM: 0.63±0.62 g and AUC: 166.9±76.6 g min^{-1}; n=9, Two-Way ANOVA P<0.001). The NaHS EC_{50} on AUC and tone increase (obtained with the maximum dose of carbachol (10 µM)) were 1.9 mM (log EC_{50}= -2.72±0.08) and 0.9 mM (Log EC_{50} =-3.00±0.09) respectively (Figure 4). After 30 minutes of NaHS 3 mM washout (maximal concentration used), the increase in tone and AUC was recovered to 107.9±2.1 % and 102.0±6.8 % from the previous control administration of carbachol 10 µM respectively (n=4, data not shown).

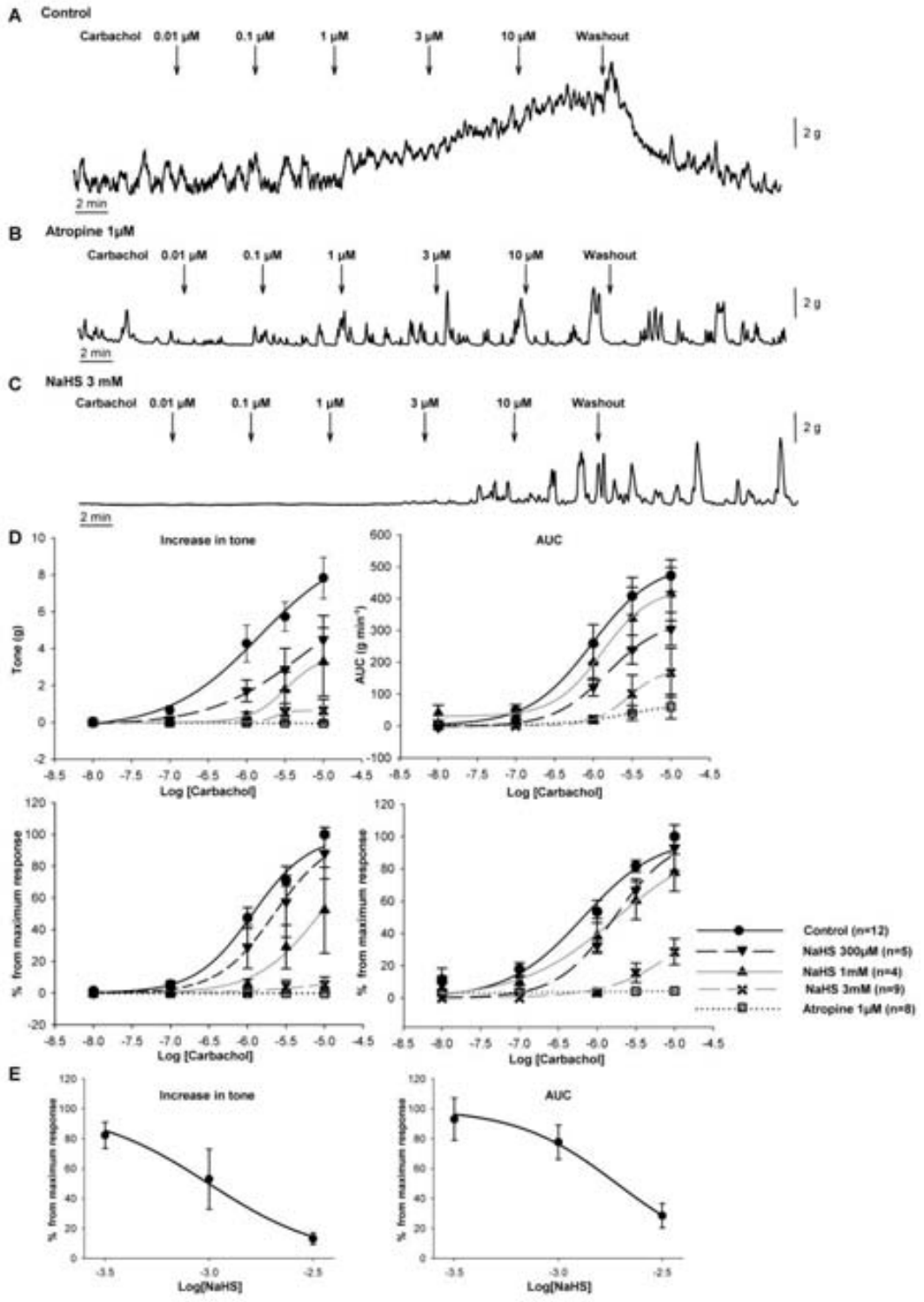

Figure 4. Mechanical recordings showing the increase in tone and AUC elicited by cumulative concentrations of carbachol in control conditions (A), and the reduction of the response after incubation with atropine (1 µM) (B) and after NaHS (3 mM) (C). (D) Graphical plots showing the effects of NaHS (300 µM, 1 mM and 3 mM) and atropine (1 µM) on the increase in tone (left) and AUC (right) elicited by additive concentration-response curve of carbachol. Plot graphs are expressed in raw data (up) and as a percentage from the maximal response obtained after 10 µM of carbachol (down) (Two-Way ANOVA P<0.001). (E) Inhibitory effect of NaHS (300 µM, 1 mM and 3 mM) on the increase in tone (left) and AUC (right) elicited by exogenous addition of carbachol (10 µM). The EC_{50} values were 0.9 mM for tone increase and 1.9 mM for AUC.

Carbachol-induced increase in AUC and tone is inhibited by NaHS in calcium free medium

The effect of NaHS on carbachol-induced responses was studied in calcium free Krebs. In these experimental conditions, increase in tone after carbachol addition (0.1 to 10 µM) was only observed in 7 of 22 strips studied, whereas AUC increase was produced in all cases, reaching at the maximum concentration of carbachol tested (10 µM) a value of 329.2±49.9 g min^{-1}. No statistically significant differences were found when compared to AUC increase obtained in normal Krebs (471.8±50.2 $gmin^{-1}$) (T test n.s.). As in non-calcium free conditions, carbachol-induced response was blocked by atropine (1 µM). NaHS significantly inhibited carbachol-induced increase in AUC, being almost completely abolished after incubation with NaHS 3 mM (EC_{50}=0.1 mM; $LogEC_{50}$=-3.95±0.67; Two-Way ANOVA P<0.001; n=7; data not shown).

EFS-induced tachykinergic contraction is inhibited by previous incubation with NaHS.

Since EFS-induced (50 V, 50 Hz, 0.4 ms, 1 s train duration) cholinergic contraction was significantly inhibited after incubation with NaHS, the tachykinergic component of the nerve mediated contraction was also studied. Previous data suggest that long-lasting trains of EFS cause the release of tachykinins (Maggi, 2000). In the human sigmoid colon, the tachykinergic response is mainly mediated by NK_2 receptors (Cao *et al.*, 2000;Auli *et al.*, 2008). In "non-nitrergic, non-purinergic" conditions and after incubation with atropine 10 µM, the EFS-induced (1s) cholinergic excitatory response was completely blocked (data not shown, n=5). When the same parameters of EFS were applied but the total train duration was increased to 10 s, a GR159897-sensitive contractile response was observed (EC_{50}=67.3 µM, $LogEC_{50}$= -4.17±0.17, n=4) suggesting a NK_2 receptor mediated response (Figure 5A and B). In these experimental conditions, tissue incubation (15 minutes) with additive concentrations of NaHS significantly reduced EFS-induced contractions (EC_{50}= 5.7 mM, $LogEC_{50}$=-2.25±0.19, Two-Way ANOVA P<0.001, n=5, Figure 5C and D). The reduction of the nerve mediated tackykinergic response was significant at 10 mM of NaHS, being the amplitude of the contraction reduced to 1.9±0.5 g (Figure 5C and D). At the end of the protocol and after NaHS washout, the amplitude of the EFS-induced contraction recovered progressively, being 85.09±12.59% from control contraction 30 minutes after washout (n=5, data not shown).

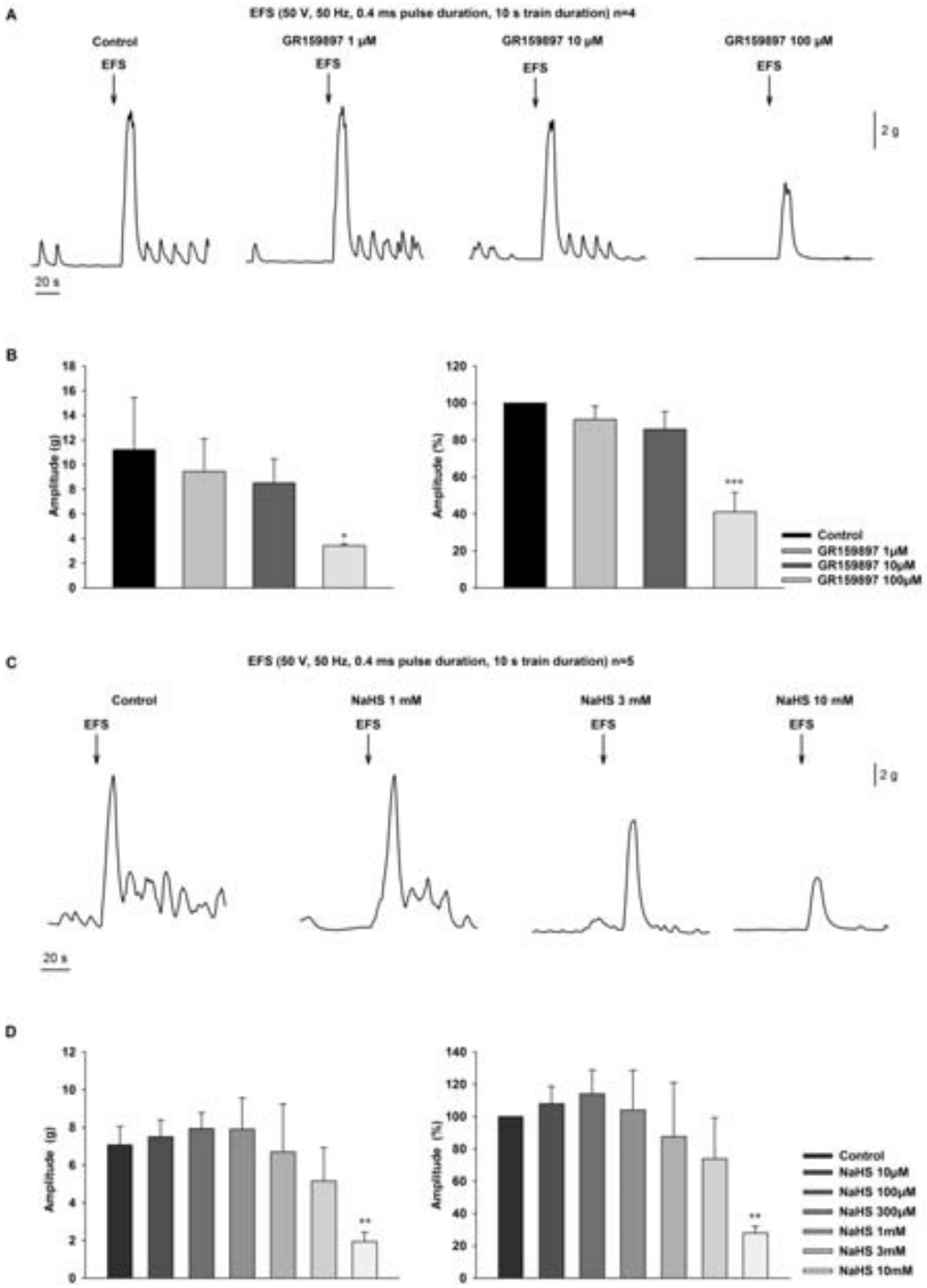

Figure 5. (A) Mechanical recordings and (B) histograms showing the reduction of the atropine-resistant EFS-induced contraction after incubation with GR159897 (1, 10 and 100 µM). (C) Mechanical recordings showing the atropine-resistant EFS-induced contraction in control conditions and after incubation with NaHS (1, 3 and 10 mM). (D) Histograms representing the effects of additive concentrations of NaHS (10 µM to 10 mM) on the atropine-resistant contraction. In the histograms, data are expressed as raw data (left) and percentage from control contraction (right). One-way ANOVA was performed followed by a Bonferroni's multiple comparison test (**P<0.01).

NKA-induced responses are inhibited by NaHS.

Exogenous administration of NKA (cumulative concentration-response curve from 0. 1 to 100 nM) caused a progressive increase in spontaneous myogenic activity (AUC) as well as an increase in tone, reaching a maximum at 100 nM of NKA (290.8±40.9 g min^{-1} and 3.8±0.5 g respectively; n=7). NKA-induced responses were totally blocked by GR159897 100 µM (n=5). Previous incubation with NaHS (0.3, 1 and 3 mM) reduced NKA contractile responses in a concentration-dependent manner. NaHS concentration-dependently inhibited the increase in tone and AUC elicited by NKA (Two-way ANOVA $P<0.001$). NaHS 3 mM totally abolished the increase in tone and also inhibited the increase in AUC (12.19±6.17 g min^{-1}; n=6) elicited by NKA cumulative concentration response curve. EC_{50} values for NaHS inhibitory effects on AUC and tone increase were 1.7 mM (log EC_{50}= -2.78±0.13) and 0.7 mM (log EC_{50} =-3.18±0.07) respectively (data obtained with NKA 0.1 µM) (Figure 6). At the end of the experimental protocol, 30 minutes after washing out NaHS (3 mM), the increase in tone and AUC elicited by exogenously added NKA 0.1 µM was 28.9±13.8 % and 65.6±6.2 % from control administration respectively (n=5, data not shown).

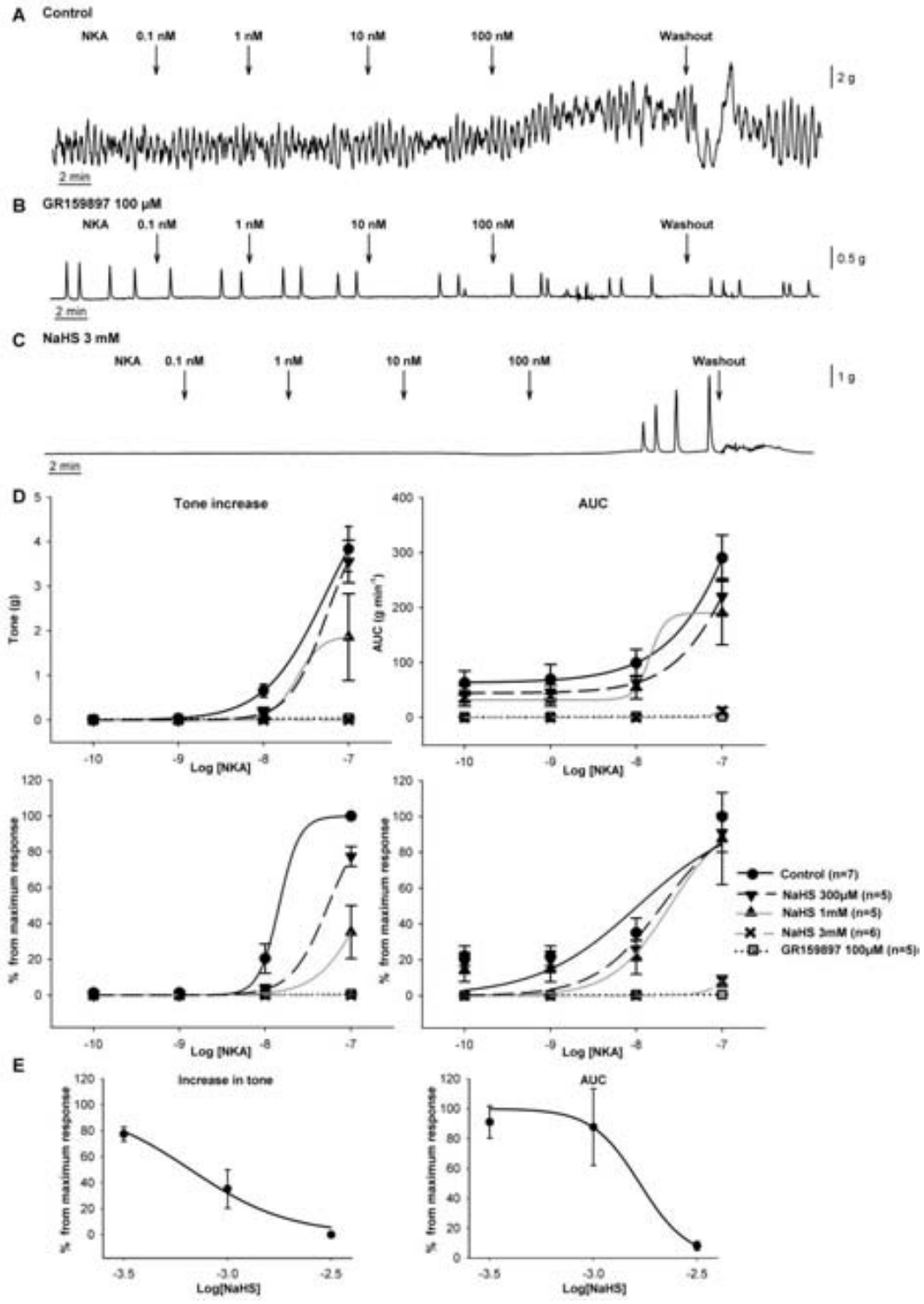

Figure 6. Mechanical recordings showing the increase in tone and AUC elicited by additive concentrations of NKA (0.1 to 100 nM) in control conditions (A) after incubation with the NK_2 antagonist GR159897 (100 µM) (B) and NaHS (3 mM) (C). (D) Graphical plots showing the effects of GR159897 and NaHS (300 µM, 1 mM and 3 mM) on the increase in tone (left) and AUC (right) elicited by cumulative concentration-response curve of NKA. Plot graphs are expressed in raw data (up) and percentage from the maximum response observed after administration of the maximum dose of NKA (100 nM) (down). Two-Way ANOVA P<0.001. (E) Inhibitory effect of NaHS (300 µM, 1 mM and 3 mM) on the increase in tone (left) and AUC (right) elicited by exogenous addition of NKA (0.1 µM). The EC_{50} values were 0.9 mM for tone increase and 1.9 mM for AUC.

DISCUSSION

In the present study we have characterized the effects of the H_2S donor NaHS on spontaneous mechanical activity and EFS-induced contractions, as well as CBS and CSE inhibitors on RMP and contractility in the human colon. The present paper provides more evidence about the inhibitory effects of endogenously synthesized H_2S and suggests a complex interaction of H_2S with NO, as well as with the excitatory cholinergic and tachykinergic pathways in the human colonic smooth muscle.

The blockade of the enzymes responsible for H_2S synthesis gives information about the putative role of constitutively produced H_2S. Our study shows that a combination of PAG 2 mM (a CSE inhibitor) and AOAA 1 mM (a CBS inhibitor) causes depolarization of RMP and a transient tone increase in the human colon. These findings suggest a constitutive production of H_2S in the human colon, as previously reported in the rat (Gil *et al.*, 2011) and mouse colon (Linden *et al.*, 2008), that contributes to the maintenance of the smooth muscle inhibitory tone. Indeed, endothelial SMCs of CSE knockout (KO) mice have a more depolarized RMP when compared to wild type (WT) animals (Tang *et al.*, 2013). Interestingly, in the mouse colon, endogenous H_2S participates in the CO-dependent transwall gradient in RMP (Sha *et al.*, 2014). Moreover, PAG depolarizes the tissue in WT animals when NO synthesis blockade (L-NNA) has been performed or in nNOS KO mice, suggesting that endogenous H_2S might be exerting an inhibitory effect on NO production (Sha *et al.*, 2014). Although we have not performed experiments to analyze the effect of endogenous H_2S production in the transwall gradient in the human colon, our results and those previously reported in rodents (Gil *et al.*, 2011;Sha *et al.*, 2014) suggest that endogenous H_2S has hyperpolarizing function. HA is another commonly used inhibitor of CBS. In our study, HA inhibited spontaneous contractions by a "NO-like effect" since it was significantly antagonized by the GC inhibitor ODQ. This is a very interesting response since HA has been considered a NO-generating compound (Iversen *et al.*, 1994;Correia *et al.*, 2000) and therefore may not be a suitable tool to block H_2S production due to its possible unselective effects. Another possible explanation for this result is that blockade of H_2S synthesis by HA might lead to an enhanced endogenous NO production (Kubo *et al.*, 2007;Sha *et al.*, 2014) which, in turn, might be causing inhibition of spontaneous motility.

Similarly to previous findings observed both in human and rat colonic strips (Gallego *et al.*, 2008a;Gil *et al.*, 2013), NaHS concentration-dependently inhibited spontaneous contractility in the presence of TTX (EC_{50}=329.2 μM), suggesting that the mechanism involved in NaHS-induced relaxation is smooth muscle mediated. Interestingly, ODQ 10 μM and L-NNA 1 mM reduced H_2S inhibitory effect, suggesting a possible synergy with the nitrergic pathway as

it has been previously proposed in other works (Hosoki *et al.*, 1997;Teague *et al.*, 2002), or even an interaction with nitrergic neuronal inputs. Therefore, the effects of H_2S may be complex and multiple. In one side, H_2S might be causing post-junctional hyperpolarization and relaxation due to a synergy with NO, but opposite to this, H_2S might also be pre-junctionally inhibiting NO production (Kubo *et al.*, 2007). Then, the net effect will result from the balance reached by these two opposite effects. Nevertheless, no effects were observed on the purinergic component of the IJP or in the EFS-induced relaxation, which is both purine and NO mediated.

In human colonic tissue, spontaneous contractions are myogenic (TTX insensitive) and independent of a neural input. Electrical stimulation often causes a transient inhibition followed by an off-response (Auli *et al.*, 2008). In order to avoid contractile responses due to rebound contractions and to isolate the excitatory component of the contractions, we used "non-nitrergic, non-purinergic" conditions. Under these experimental conditions, EFS (frequency 50 Hz and total duration 1 s, which represent 50 pulses) caused a cholinergic (atropine sensitive) response, whereas a tachykinergic response (GR159897 sensitive and atropine insensitive) was elicited when longer durations of EFS were applied (frequency 50 Hz and total duration 10s, which represent 500 pulses). Previously published studies have reported H_2S inhibitory effects on cholinergic responses in guinea-pig ileum (Teague *et al.*, 2002), murine airway smooth muscle (Castro-Piedras & Perez-Zoghbi, 2013) and in rat colon (Gil *et al.*, 2013) preparations. Similarly, our results demonstrate that NaHS is able to inhibit neural mediated cholinergic (EC_{50}= 2 mM) and tachykinergic (EC_{50}= 5.7 mM) contractions in the human colon, although the concentration needed to reduce neural mediated excitatory responses was higher than the concentration needed to reduce spontaneous motility (EC_{50}= 329.2 μM).

In the present work we also demonstrate that NaHS inhibits the cholinergic/tachykinergic contractile responses induced both by EFS and by exogenous addition of carbachol/NKA respectively. The inhibitory effect on EFS-induced responses can be pre or post-junctionally exerted. Preliminary experiments performed in rat longitudinal myenteric plexus preparation have reported an increase of ACh release after NaHS administration (not shown), suggesting that incubation with NaHS is not reducing ACh production. Due to the fact that NaHS reduces spontaneous contractions in the presence of TTX, and also inhibits the contractile response evoked by exogenously added carbachol or NKA, our results suggest a post-junctional inhibitory effect. Since NaHS is a hyperpolarizing mediator (possibly acting on K^+ channels (Gil *et al.*, 2013;Gallego *et al.*, 2008a)) the reduction of neural

mediated excitatory responses might be due to the hyperpolarization itself, which is able to decrease the probability of opening L-Type calcium channels. Another concern is that NaHS was also able to block the carbachol-induced contractile response in calcium-free medium. Accordingly, it is also possible that H_2S might be reducing post-junctional intracellular pathways independently of the smooth muscle RMP. According to this hypothesis it has been recently demonstrated that H_2S inhibits calcium release through IP_3 receptors (Castro-Piedras & Perez-Zoghbi, 2013), which might constitute another possible mechanism involved in the reduction of excitatory pathways.

The frontier between physiological, pharmacological and toxic effects of H_2S remains unclear. The reported physiological concentration of H_2S in human blood is approximately 100 µM (Teague *et al.*, 2002). In the present work, higher H_2S concentrations have been needed in order to significantly block spontaneous contractions (EC_{50}=329.2 µM) and EFS-induced cholinergic or tachykinergic contraction (EC_{50}= 2 mM and 5.7 mM respectively). Despite these relatively high concentrations, our results demonstrate that H_2S inhibitory effects are at least in part reversible, so they may not be related to an irreversible impairment of tissue function due to toxicity. Even if H_2S concentration in the external medium is high, concentration inside the tissue may be much lower, or even trivial (Linden *et al.*, 2010). Thus, at the moment it seems difficult to determine the real concentration of H_2S which is exerting the effects inside the tissue, the physiological relevance of H_2S actions or the associated potential therapeutic applications.

In the present study we have provided experimental evidence about the putative role of H_2S as an endogenous inhibitory signalling molecule in the human colon. H_2S could elicit direct inhibitory effects on human colonic smooth muscle possibly in synergy with NO. We have also demonstrated that H_2S may be exerting its inhibitory effects on colonic contractility by decreasing neural mediated cholinergic and tachykinergic excitatory pathways, possibly at the post-junctional level. Further studies are needed in order to investigate the potential role of H_2S in pathophysiological conditions in the human GI tract associated with colonic hypomotility and to determine the potential role of H_2S (alone or in combination with NO donors) as a pharmacological target to inhibit motility.

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CHAPTER 4

Mechanisms of action of Otilonium Bromide (OB) in human cultured smooth muscle cells and rat colonic strips

Neurogastroenterology and Motility 25, e803-e812 (2013)

ABSTRACT

In the present work we investigated the different pharmacological properties of the spasmolytic drug Otilonium Bromide (OB). Human cultured colonic smooth muscle cells (HCSMCs) were studied using the calcium imaging technique. Microelectrodes and muscle bath experiments were performed in rat colonic strips. OB concentration-dependently inhibited nifedipine sensitive calcium transients induced by KCl (EC_{50}=3.56 μM) and BayK8644 (EC_{50}=3.99 μM). $CaCl_2$ induced nifedipine sensitive contractions in colonic strips incubated in nominally calcium-free Krebs. $CaCl_2$ response was inhibited by OB (EC_{50}= 4.88 μM). To avoid a possible interference with L-type calcium channels all the following experiments were performed in the presence of nifedipine. In HCSMCs, carbachol-induced calcium transients were inhibited by OB (EC_{50}=8.45 μM). Carbachol evoked 1- a smooth muscle depolarization (10 mV) that was antagonised by 100 μM OB; and 2- a contraction that was inhibited by OB (EC_{50}=13 μM). "Non-nitrergic (L-NNA 1 mM) non-purinergic (MRS2500 1 μM)" conditions were used to elicit endogenous excitatory responses: Electrical field stimulation caused an atropine-sensitive excitatory junction potential that was concentration-dependently inhibited by OB (EC_{50}= 8.87 μM) and an atropine sensitive contraction that was concentration-dependently inhibited by OB (EC_{50}=7.28 μM). In HCSMCs, neurokinin A (NKA) and $CaCl_2$ induced calcium transients that were inhibited by OB (NKA: EC_{50}=11.70 μM; $CaCl_2$: EC_{50}=17.50 μM). OB causes inhibition of L-/T-type calcium channels, muscarinic and tachykininergic responses that acting together explain the pharmacological properties of the compound.

INTRODUCTION

Otilonium Bromide (OB) is a spasmolytic agent used to treat gastrointestinal (GI) motor disorders. OB reduces pain and discomfort (Battaglia *et al.*, 1998) and enhances sensory thresholds to recto-simoidal distension (Czimmer *et al.*, 2001) in patients with irritable bowel syndrome (IBS). In a recent randomized clinical trial it has been demonstrated that OB reduces the frequency of abdominal pain and severity of abdominal bloating in IBS patients and interestingly, its beneficial activity is observed also when stopping treatment (Clave *et al.*, 2011). OB is considered a "musculotropic spasmolytic" drug, which probably acts directly on intestinal smooth muscle cells (SMCs) reducing contractility (Boeckxstaens *et al.*, 2013) and possess a peculiar selective activity on the gut being not systemically absorbed (Evangelista *et al.*, 2000). Several pharmacological properties have been attributed to OB. OB is an L- and T-type calcium channel blocker and also has anti-muscarinic and anti-tachykininergic properties (Santicioli *et al.*, 1999;Martin *et al.*, 2004;Strege *et al.*, 2010;Strege *et al.*, 2004;Gallego *et al.*, 2010). OB binds to L-type calcium channels, NK_2 and muscarinic receptors including those from the rat colon (Evangelista *et al.*, 1998;Santicioli *et al.*, 1999). Using the patch-clamp technique, it has been shown that OB inhibits L-type calcium channels in SMCs from human jejunum (Strege *et al.*, 2004) and rat colon (Martin *et al.*, 2004). Moreover OB also inhibits T-type calcium channels (Cav 3.1, Cav 3.2 and Cav 3.3) expressed in HEK293 cells (Strege *et al.*, 2010). Calcium transients induced by KCl in isolated human cultured colonic smooth muscle cells (HCSMCs) were also concentration-dependently inhibited by OB (Gallego *et al.*, 2010). Accordingly, inhibition of voltage dependent Ca^{2+} channels (VDCC) may underlie the inhibition of the spontaneous motility observed in vitro both in human and rat colonic strips (Gallego *et al.*, 2010;Martin *et al.*, 2004). Consistent with the binding data, OB inhibits acetylcholine (ACh) and tachykinins (TKs) induced contractions due to the activation of excitatory motor neurons in human colonic strips (Gallego *et al.*, 2010). All these pharmacological properties are probably crucial to understand the musculotropic spasmolytic effects of OB.

It is well known that mechanisms leading to contractile responses are related. Excitatory motor neurons release ACh and TKs that activate post-junctional receptors leading second messengers that activate non-selective cation channels that can contribute to calcium entrance. Activation of non-selective cation channel causes smooth muscle depolarization that activates spiking activity due to opening of VDCC causing contraction (Sanders *et al.*, 2012). Probably, one of the most interesting properties of OB that explains the musculotropic spasmolytic effect of the drug is the combination of different mechanism of actions that might act in synergy. However, it is important to try to discriminate between putative effects due to

inhibition of each mechanism. The pharmacological properties of OB have been investigated using different experimental models, techniques and conditions and consequently the results are not always easy to compare. Accordingly, the aim of the present work was to investigate and possibly isolate the pharmacological properties of OB in HCSMCs which is the main target of the drug "in vivo". Rat colonic strips (Martin *et al.*, 2004) were used to confirm the pharmacological properties. Preliminary data of the present work were presented at the II Joint Neurogastroenterology and Motility Meeting (Bologna, Italy, 2012) and published in abstract form (Jimenez *et al.,* 2012).

MATERIALS AND METHODS

Rat tissue preparation

Male Sprague-Dawley rats (8–10 week old, 300–350 g) were purchased from Charles River (Lyon). Animals were housed under controlled conditions: temperature 22ºC ± 2ºC, humidity 55% ± 10%, 12:12-h light-dark cycle and access to water and food *ad libitum*. Animals were killed by decapitation and exsanguination. This procedure was approved by the Ethics Committee of the Universitat Autònoma de Barcelona, Bellaterra, Spain.

After opening the abdominal cavity, the colon was removed 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 to a Sylgard base with the mucosa facing upward. The mid colon was distinguished according to the anatomical criteria previously described (Alberti *et al.*, 2005). Mucosal and submucosal layers were carefully removed and circular or longitudinal muscle strips were cut into strips.

Intracellular microelectrode recording

Samples were pinned with the circular muscle layer facing upward in a Sylgard-coated chamber, continuously perfused with carbogenated Krebs solution at 37ºC ± 1ºC. Tissue was allowed to equilibrate for 1 hour before starting the experiment. Circular SMCs were impaled with glass microelectrodes filled with 3 M KCl (30-60 MΩ of resistance). Membrane potential was measured by using 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). Experiments were performed in the presence of nifedipine (1 μM) to stabilize impalements. Electrical field stimulation (EFS) was applied using two silver chloride plates placed perpendicular to the longitudinal axis of the preparation and 1.5 cm apart. Train stimulation had the following parameters: total duration, 100 ms; frequency, 30 Hz; pulse duration, 0.3 ms, and increasing amplitude voltage (8, 12, 16, 20, 24, 28, 32, 36 and 40 V). Inhibitory junction potential (IJP) was elicited by EFS using single pulses and excitatory junction potential (EJP) was evaluated using the same stimulation parameters in the presence of L-NNA (1 mM) and MRS2500 (1 μM) to block nitrergic and purinergic neuromuscular transmission respectively. Resting membrane potential (RMP) (expressed in mV) was measured before and after drug addition. The amplitude (mV) of IJP and EJP was measured under control conditions and after infusion of each drug infusion.

Mechanical studies

Strips (1 x 0.3 cm) were mounted in a 10 mL organ bath containing carbogenated physiological saline solution maintained at 37 ± 1ºC. Contractions from the circular and longitudinal muscle layer were measured using an isometric force transducer (Harvard VF-1 Harvard Apparatus Inc., Holliston, MA, USA) connected to a computer through an amplifier. Data were digitized (25 Hz) using Data 2001 software (Panlab, Barcelona, Spain) coupled to an A/D converter installed in the computer. A tension of 1 g was applied and tissues were allowed to equilibrate for 1 h after which strips displayed spontaneous phasic activity. EFS was applied (pulse duration 0.3 ms, frequency 50 Hz, and amplitude 50 V; total duration 1 s). The amplitude of contractions from the baseline (maximum-minimum) and the area under curve (AUC) were used to evaluate the EFS or drug-induced contractions.

Cell culture and calcium imaging technique

Human cultured colonic smooth muscle cells (HCSMCs) (Innoprot, Bizkaia, Spain) were studied by using the calcium imaging technique. Cells were grown in Smooth Muscle Cell Medium kit (37ºC pH 7.4) (Innoprot, Bizkaia, Spain) and seeded in a culture dish with a coverglass 48h before the experimental procedure. Cells were loaded with Fluo-4AM (5 µM) and pluronic acid (25% DMSO 2 µL mL^{-1}) at room temperature, for 55 min in extracellular medium solution (containing, in mM: 140 NaCl, 4.8 KCl, 1 $MgCl_2$ $6H_2O$, 1.8 $CaCl_2$ $2H_2O$, 10 glucose). The coverglass containing the Fluo-4AM loaded cells was placed over a metal ring, immobilized by a rubber o-ring and transferred to a chamber. Cells were imaged with IX-FLA Camera (Olympus Biosystems, Heidelberg, Germany) connected to an Olympus IX70 microscope and scanned using Cell^R software (Olympus Biosystems). Cells were perfused in a constant flow of extracellular medium solution (1 mL min^{-1}) and a manual valve ALA VM-8 Channel Bath Perfusion System (npi Electronic Instruments, Tamm, Germany) allowed switching between normal and drug-containing solutions. Before starting the experiments, extracellular medium was perfused during 5 minutes to washout extracellular Fluo-4AM. The experimental protocol consisted of two consecutive administrations (15 seconds) of an agonist. In-between plates were incubated during 10 minutes with extracellular medium and the corresponding antagonist or its vehicle (control) (Figure 1). The same concentration of antagonist/vehicle was present during and after the second perfusion of the agonist to avoid its possible washout. Both administrations of the agonist were done during a 45-second movie and changes in Fluo4 fluorescence were recorded at 2.5Hz with a spatial resolution of 512 x 480 pixels. Only one protocol (two movies) was performed in each plate. At the end of the

experiments images were analysed over time using regions of interest (ROIs). Fluorescence increase (ΔF) of the ROIs analysis was performed and plotted over time. In order to normalize data, ΔF was calculated using the following formula: [(maximum-minimum)/minimum] x100. The minimum was obtained by calculating the mean of the fluorescence values of the 10 first frames (first 4 seconds of the movie). The ratio ΔF2/ΔF1 was calculated with two consecutive responses (Figure 1). Vehicle (control) and antagonists incubated between responses were compared.

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 following drugs were used: otilonium bromide (OB) (Menarini Group, Florence, Italy), nepadutant (American Custom Chemicals, San Diego, CA, USA), GR159897 (Santa Cruz Biotechnology, San Diego, CA, USA), carbachol (carbamoylcholine chloride), N^{ω}-nitro-L-arginine (L-NNA), BayK8644, atropine sulphate, neurokinin A (NKA), mibefradil dihydrochloride hydrate, nifedipine (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 diammonium salt (MRS2500) (Tocris, Bristol, UK), Fluo-4/AM, pluronic acid (Invitrogen, Paisley, UK). Stock solutions were made by dissolving drugs in distilled water except for nifedipine and BayK8644 which was dissolved in 96% ethanol (0.01% final concentration). L-NNA, which was dissolved in physiological saline solution by sonication and nepadutant, GR159897, Fluo-4AM and pluronic acid which were dissolved in DMSO.

Data analysis and statistics

Mechanical and electrophysiological experiments were assessed by Paired student's t-test or One-way ANOVA followed by a Bonferroni's multiple-comparison test. Ratios (ΔF2/ΔF1) were compared using One-way ANOVA followed by a Bonferroni post-hoc tests. For each drug 40 cells from at least 4 different plates were analyzed. Data are expressed as mean ± S.E.M. A $P < 0.05$ was considered to indicate statistical significance. "n" values indicate the number of animals or HCSMCs. IC_{50} was calculated using a conventional sigmoid concentration-response curve with variable slope. Statistical analysis and curve fitting were performed with GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA, USA).

RESULTS

Otilonium Bromide as an L-type calcium channel inhibitor.

KCl 75 mM induced calcium transients in HCSMCs. The response was inhibited by nifedipine (1 µM) (One-Way ANOVA $p<0.001$). OB concentration-dependently inhibited calcium transients induced by KCl ($logEC_{50}$=-5.45±0.16, EC_{50}=3.56 µM; n=290 cells; One-Way ANOVA $p<0.001$; Figure 1).

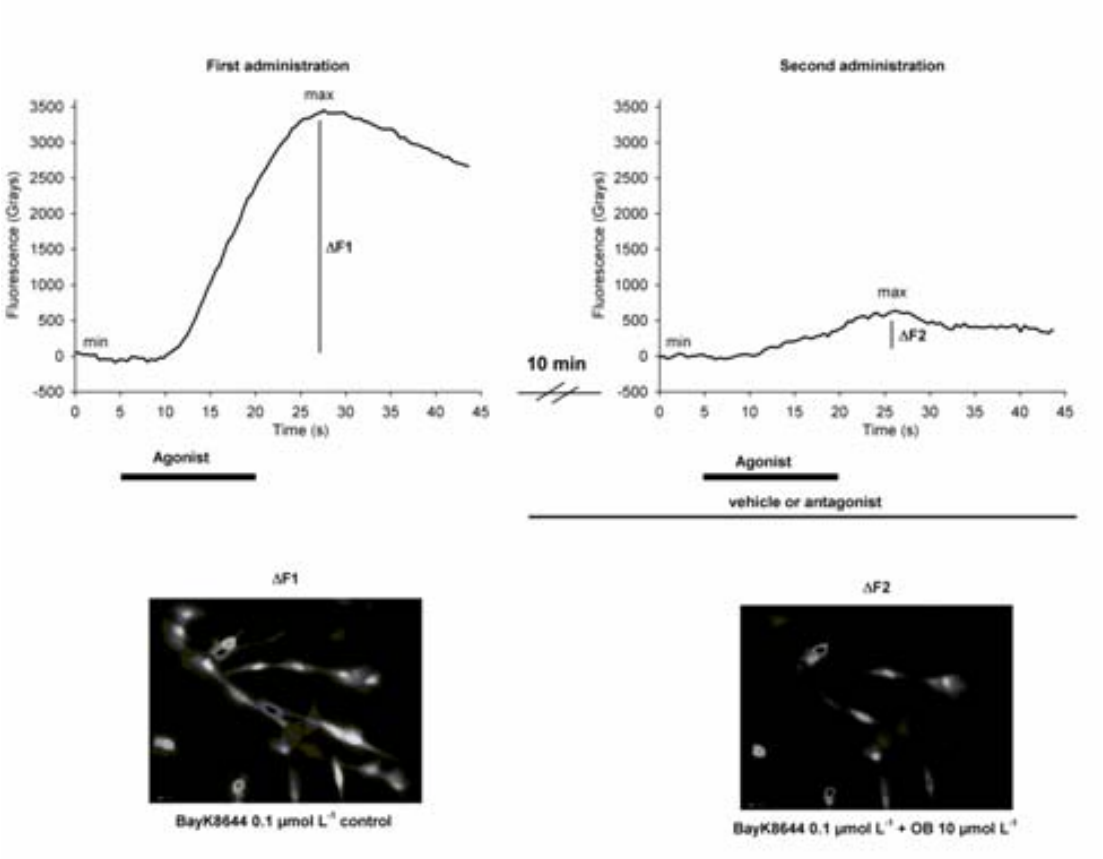

Figure 1. Plot graphs illustrating the protocol performed in HCSMCs studied with calcium imaging. Two consecutive administrations of the agonist and their corresponding movies were recorded with the same plate. Between both administrations cells were incubated during 10 minutes with an antagonist (or its vehicle as a control). Regions of interest (ROIs) were delimited. The response elicited by the agonist is quantified as increase in fluorescence or ΔF (ΔF = frame maximum- frame minimum). ΔF1 corresponds to the first administration (left) and ΔF2 to the second administration (right). The ratio ΔF2/ ΔF1 was calculated for statistical purposes. Each ΔF is illustrated in the pictures below each graph. ΔF corresponds to the subtraction of two images from the same movie using Adobe Photoshop CS2. White color illustrates ΔF whereas black color indicates the same level of fluorescence.

The L-type calcium channel agonist BayK8644 0.1 µM induced calcium transients in HCSMCs that were reduced by nifedipine (1 µM) and concentration-dependently inhibited by OB ($logEC_{50}$=-5.39±0.14, EC_{50}=3.99 µM; n=146 cells; One-Way ANOVA p<0.001; Figure 2).OB also inhibited muscular contraction caused by $CaCl_2$ (10 mM) in rat colonic strips (Figure 2). To reveal the effect of OB on L-type calcium channels, we incubated the tissue in nominally calcium free Krebs. Under these conditions addition of $CaCl_2$ (10 mM) caused a response consisting of a first contraction followed by spontaneous rhythmic contractions (Figure 2). The response was nifedipine sensitive demonstrating that the contraction was due to activation of L-type calcium channels. OB caused a concentration-dependent inhibition of the first contractile response ($logEC_{50}$=-5.31±0.18, EC_{50}=4.88 µM; n=6; One-Way ANOVA p<0.001; Figure 2). Exactly the same results (not shown) were obtained if the effect of OB on the AUC was measured. These results demonstrate that OB is an L-type calcium channel inhibitor both in HCSMCs and rat colonic strips.

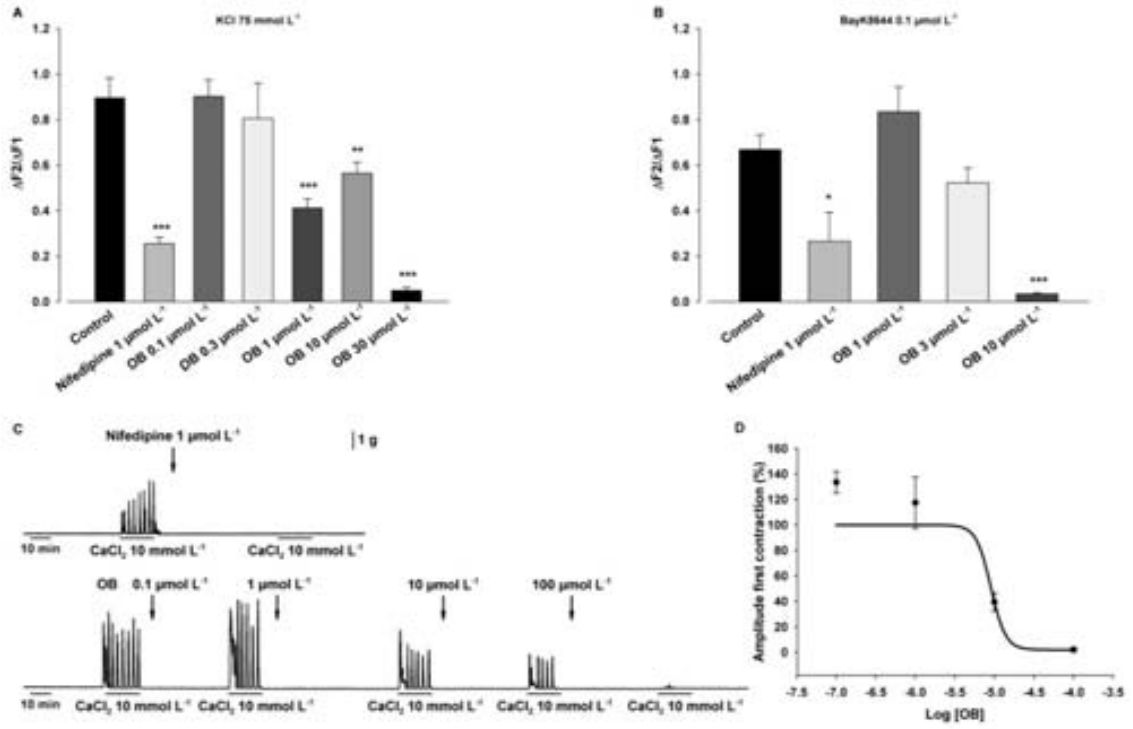

Figure 2. Calcium transients induced in HCSMCs by KCl (75 mM) **(A)** and BayK8644 (0.1 µM) **(B)** in control conditions and after incubation with nifedipine (1 µM) and different concentrations of OB. **(C)** Addition of $CaCl_2$ (10 mM) in rat colonic strips incubated with calcium nominally free Krebs in control conditions and after incubation with nifedipine (1 µM) (top) and additive doses of OB (bottom). **(D)** Dose-response curve corresponding to the OB effect on the fist contraction elicited by $CaCl_2$ (10 mM). One-way ANOVA was performed followed by a Bonferroni's multiple comparison test * p<0.05, ** p<0.01, ***p<0.001.

Otilonium Bromide as a muscarinic antagonist.

Carbachol 1 µM induced calcium transients in HCSMCs that were reduced by atropine 5 µM and concentration-dependently inhibited by OB ($logEC_{50}$=-5.54±0.28, EC_{50}=2.83 µM; n=161 cells; One-Way ANOVA p<0.001; Figure 3). This increase in calcium might be due to 1- release of calcium from intracellular stores and 2- smooth muscle depolarization (see below RMP studies) and opening of L-Type calcium channels. Accordingly, it is difficult to establish if the inhibition of the response is due to an effect on muscarinic receptors or to an effect on the L-type calcium channel (see previous results). To block L-type calcium channels, experiments were performed in the presence of nifedipine (1 µM). In the presence of nifedipine, carbachol (1 µM) induced calcium transients in HCSMCs that were concentration-dependently inhibited by OB ($logEC_{50}$=-5.07±0.30, EC_{50}=8.45µM; n=170 cells; One-Way ANOVA p<0.01; Figure 3). These results suggest a direct effect of OB on the cholinergic pathway.

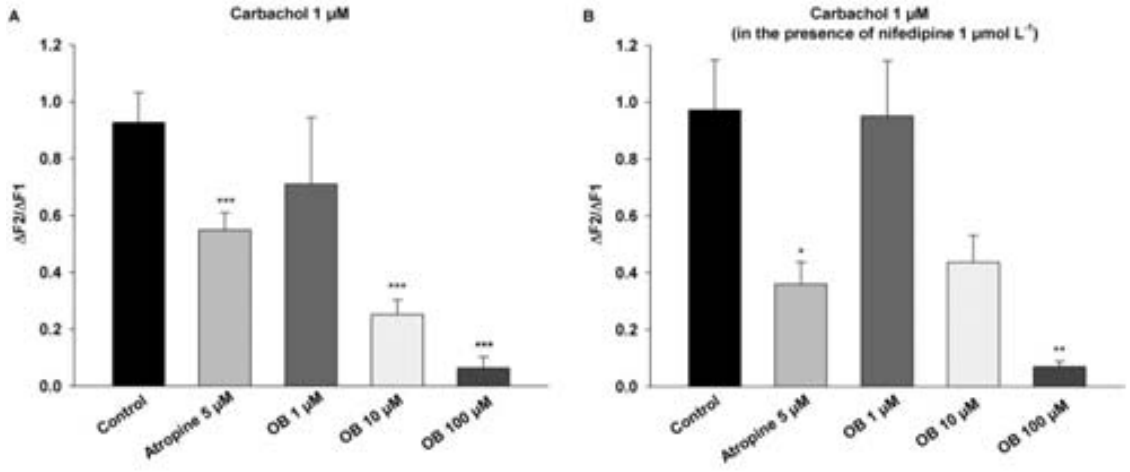

Figure 3. Calcium transients in HCSMCs induced by carbachol (1 µM) in control conditions and after incubation with atropine (5 µM) and OB (from 1 to 100 µM). Experiments were performed using normal Krebs (**A**) and in the presence of nifedipine (1 µM) (**B**). One-way ANOVA followed by a Bonferroni's multiple comparison test was performed * p<0.05, ** p<0.01, ***p<0.001.

Experiments performed in rat colonic strips incubated with nifedipine showed that carbachol (10 µM) induced a smooth muscle depolarization (10.34 ± 0.96 mV, n=4) that was inhibited by OB (100 µM) pre-incubation of the tissue (2.74 ± 1.04 mV; n=4; Paired t-test P<0.05; Figure 4). In the presence of nifedipine (1 µM), single pulse electrical field stimulation caused an IJP with two consecutive phases: a fast followed by a sustained smooth muscle hyperpolarization (Figure 4). L-NNA (1 mM) reduced the sustained IJP whereas the cumulative incubation with the $P2Y_1$ receptor antagonist MRS2500 (1 µM) revealed an excitatory junction potential (Figure 4). It is important to note that the "predominant" innervation of the colon is inhibitory and consequently the EJP can only be elicited in "non-nitrergic, non-purinergic" conditions. The EJP was completely abolished by atropine 1 µM (n=3) and concentration dependently inhibited by OB ($logEC_{50}$=-5.05±0.29, EC_{50}=8.87µM; n=6; One-way ANOVA p<0.05; Figure 4).

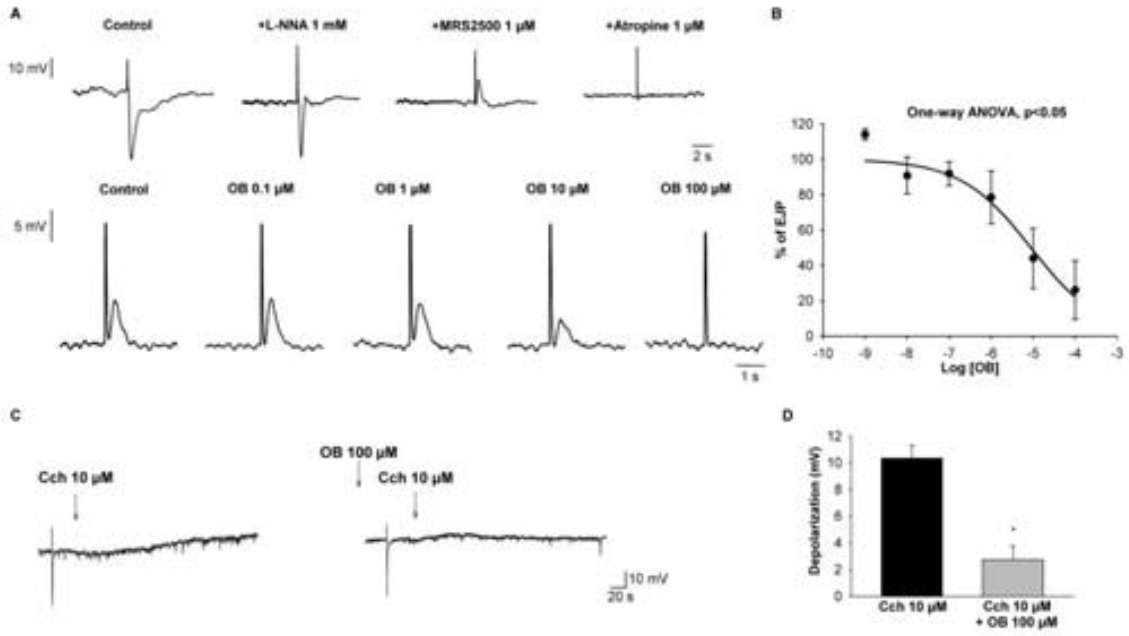

Figure 4. (A) Intracellular recordings showing the EJP elicited in rat colonic strips after the sequential incubation with L-NNA (1mM) and MRS2500 (1 µM). The EJP was blocked by atropine (1 µM) and additive concentrations of OB **(B).** Plot graph showing the concentration-response curve of OB on the EJP. One-way ANOVA followed by a Bonferroni's multiple comparison test was performed. Intracellular recording **(C)** and histogram **(D)** showing the depolarization of resting membrane potential elicited by carbachol (1 µM) in control conditions and after incubation with OB (100 µM). A paired student's t-test was performed * p<0.05.

To evaluate the effect of OB on mechanical responses tissue was incubated with nifedipine (1 µM), L-NNA (1 mM) and MRS2500 (1 µM). Under "non-nitrergic, non-purinergic" conditions both EFS (n=6) and carbachol (10 µM) (n=5) induced an atropine sensitive contraction that was probably due to calcium release from internal stores. Both EFS and carbachol induced responses (measured as amplitude and AUC respectively) were concentration-dependently inhibited by OB (EFS: $\log EC_{50}$=-5.14±0.15, EC_{50}=7.28 µM, One-way ANOVA p<0.001, n=6; carbachol: $\log EC_{50}$=-4.89±0.15, EC_{50}=13.00 µM, n=5, One-way ANOVA p<0.001, Figure 5).

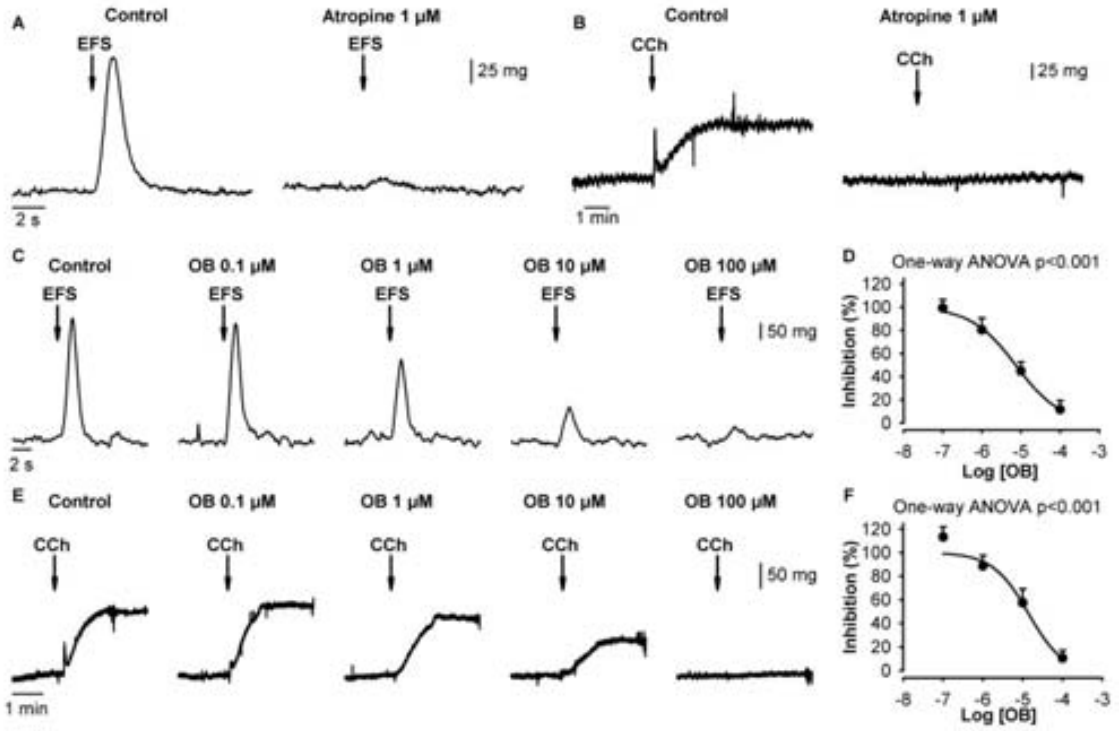

Figure 5. EFS **(A)** and carbachol **(B)** contractile effect in rat colonic strips in the presence of nifedipine (1 µM) and after incubation with atropine (1 µM). A paired student's t-test was performed. Recordings **(C)** and plot graph **(D)** showing the reduction of the amplitude of contraction elicited by EFS after incubation with additive doses of OB. Recordings **(E)** and plot graph **(F)** showing the reduction of contraction (AUC) elicited by carbachol (Cch, 10 µM) after the incubation with additive concentrations of OB. One-way ANOVA followed by a Bonferroni's multiple comparison test was performed.

Otilonium Bromide as a tachykininergic antagonist

In HCSMCs and in the presence of nifedipine, neurokinin A (NKA) (0.1 μM) induced calcium transients probably due to calcium release from internal stores. It is important to note that the second response was reduced to about 40% compared to the first response (ratio 0.6). The second response was partially reduced by the NK_2 receptor antagonists GR159897 (10 μM) and nepadutant (10 μM). The effect was concentration-dependently inhibited by OB ($logEC_{50}$=-4.93±0.24; EC_{50}=11.7 μM, n= 139 cells; One-way ANOVA p<0.001; Figure 6).

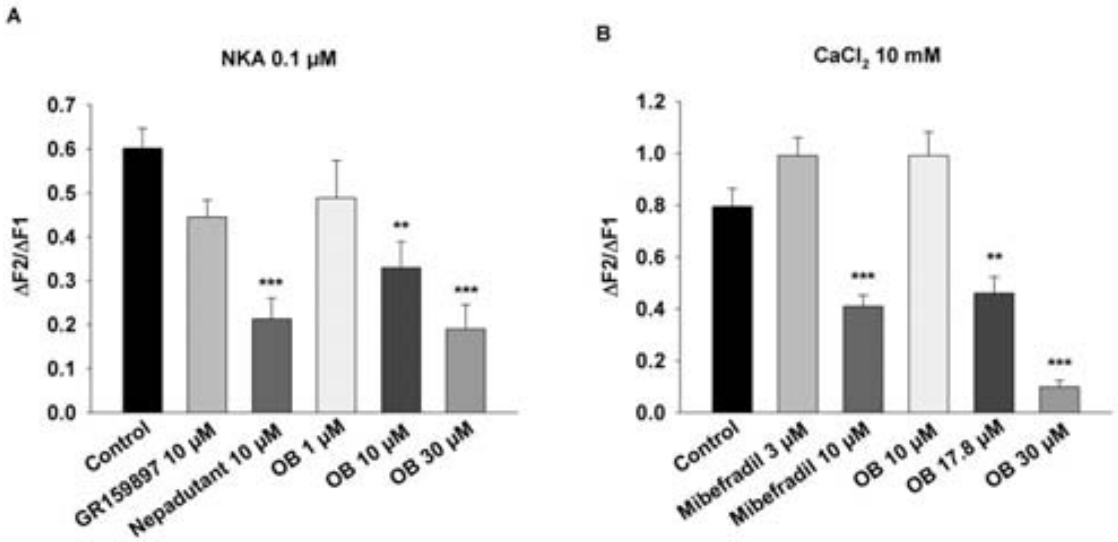

Figure 6. **(A)** Calcium transients in HCSMCs induced by NKA (0.1 μM) in control conditions and after incubation with NK_2 antagonists and cumulative doses of OB. **(B)** Calcium transients induced by $CaCl_2$ (10 mM) in control conditions and after incubation with cumulative mibefradil and OB. All experiments were performed in the presence of nifedipine (1 μM). One-way ANOVA followed by a Bonferroni's multiple comparison test was performed; ** p<0.01, ***p<0.001.

Otilonium Bromide as a T type calcium channel blocker

To reveal T-type calcium channel responses HCSMCs were incubated with nifedipine (1 μM). $CaCl_2$ (10 mM) caused a calcium transient probably due to activation of non L-type calcium channel opening at the resting state. $CaCl_2$ response was reduced by mibefradil (10 μM) (p<0.001). OB concentration-dependently reduced the response to $CaCl_2$ (10 mM) ($logEC_{50}$=-4.76±0.02; EC_{50}=17.5μM, n=160 cells; One-way ANOVA p<0.001; Figure 6).

DISCUSSION

In the present work we confirm previous data suggesting that OB is a musculotropic spasmolitic agent causing smooth muscle relaxation by inhibition of voltage gated calcium channels and inhibition of muscarinic and neurokinin receptors. The activity of OB is found in the concentration range from 1 to 10 µM where the majority of the effects of the drug were detected.

One crucial property of OB is inhibition of voltage gated calcium channels that might contribute to calcium influx in SMCs. Both L-type and T-type calcium channels are expressed in GI SMCs and contribute to contraction and pacing (Gallego *et al.*, 2008;Evangelista *et al.*, 1998;Strege *et al.*, 2010). As it was previously demonstrated with the patch-clamp technique, L- and T-type currents were inhibited by OB at a range of concentrations of about 1 µM (Gandia *et al.*, 1996;Martin *et al.*, 2004;Strege *et al.*, 2010;Strege *et al.*, 2004). Similar results were obtained in HCSMCs with calcium transient inhibition elicited with different agonists (present work). In this study we used KCl to depolarize SMCs, BayK8644 which is an L-type calcium channel opener and $CaCl_2$ (in the presence of nifedipine) to elicit T-type calcium channel mediated responses. T-type calcium channels are able to produce a window current at the resting state of many cells. Increasing extracellular calcium concentrations increases the driving force enough to produce a calcium signal that can be detected using calcium sensitive fluorescent dyes (Perez-Reyes *et al.*, 2009). Mibefradil is an L-/T-type calcium channel blocker which is usually more potent as inhibitor of T-type calcium channel and whose activity is confirmed in the present work. In fact, mibefradil inhibited calcium transients (even in the presence of 1 µM nifedipine) suggesting an involvement of T-type calcium channels in the response. It is important to note that at 1 µM nifedipine did probably not interfere with the response driven by T-type calcium channels (Perez-Reyes *et al.*, 2009). In our preparations the rank of order of the EC_{50} values was L-type<T-type which slightly differs from the results obtained from HEK cells expressing different subunits of L and T type calcium channels (Strege *et al.*, 2010;Strege *et al.*, 2004). In those experimental conditions OB was more effective blocking T than L-type calcium channels and the order of IC_{50} values was CaV3.3< CaV3.1 = CaV3.2 <CaV1.2 (Strege *et al.*, 2010;Strege *et al.*, 2004). Differences in experimental conditions might be responsible for these differences. Interestingly when we tried to reproduce the effect in strips, $CaCl_2$ response did not modify spontaneous motility in normal Krebs solution (not shown). This suggests that the increase in calcium obtained in isolated HCSMCs is not enough to modify the contractibility of strips. Only when nominally calcium free Krebs was used a $CaCl_2$ response was obtained but in this case the response was totally inhibited by nifedipine

suggesting a major involvement of L-type calcium channels in the response and the crucial role of L-type calcium channels in the development of spontaneous contractions in the colon. OB inhibits $CaCl_2$ response as also detected in spontaneous motility in rats (Martin *et al.*, 2004) and humans (Gallego *et al.*, 2010).

L-type calcium channels are voltage dependent and smooth muscle depolarization causes smooth muscle action potentials (spikes) leading intracellular calcium increase and contraction. It is therefore quite difficult to distinguish between possible effects of OB due to L-type calcium channel blocking from other possible effects of the drug. In the present work we performed experiments in the presence of nifedipine to block L-type calcium channels and therefore the effect of OB could not be attributed to their inhibition. Interestingly we found that: 1- calcium transients induced by carbachol are inhibited by OB in HCSMCs; 2- OB inhibits the carbachol-induced depolarization and contraction in rat colonic strips; 3- atropine sensitive excitatory junction potentials induced by EFS are inhibited by OB as well and finally 4- atropine sensitive contractions induced by EFS are also inhibited by OB. All these result are consistent with an effect of OB on muscarinic receptors as previously reported with binding studies (Evangelista *et al.*, 1998;Santicioli *et al.*, 1999). It is important to note that the majority of the innervation of the colon is inhibitory (Gallego *et al.*, 2011;Gallego *et al.*, 2008;Pluja *et al.*, 1999). OB does not modify IJP in the rat colon (Martin *et al.*, 2004) and does not change inhibitory mechanical response in human colonic strips (Gallego *et al.*, 2010). Accordingly, OB does not modify inhibitory pathways responsible for smooth muscle relaxation. To reveal excitatory junction potentials it is necessary to change the classical “non-adrenergic, non-cholinergic” conditions by “non-nitrergic, non-purinergic” conditions (L-NNA+MRS2500) and consequently a cholinergic EJP can be elicited even in the presence of nifedipine. The present study is not the first study to evaluate the effect of OB on the EJP. Santicioli et al. (Santicioli *et al.*, 1999) measured the effect of OB on the EJP from the guinea-pig colon using sucrose-gap technique. They found that OB reduced the EJP at concentrations above 10 µM. Our results are in the same range i.e. between 1 and 10 µM and we confirm the possibility that OB is inhibiting the effect of cholinergic excitatory neurons.

TKs are also other known excitatory mediators in the GI tract (Shimizu *et al.*, 2008). The addition of NKA to bath muscle strips increases contractions suggesting that TKs are excitatory mediators regulating contractility. NKA induced calcium transients that were inhibited by the NK_2 receptor antagonist nepadutant (Catalioto *et al.*, 1998) and OB. Similar results were described by Santicioli et al. (Santicioli *et al.*, 1999) in the guinea pig ileum.

It is important to notice that in the present work we did not investigate the possible effects of OB on Na^+ channels. Intestinal myocites express Nav1.5 channels (Xiong *et al.*, 1993;Holm *et al.*, 2002;Strege *et al.*, 2003a;Ou *et al.*, 2002) responsible for tetrodotoxin (TTX)-insensitive Na^+ currents. The exact role of these channels regulating smooth muscle contractibility is still unknown. Na^+ channels might contribute to the upstroke of the slow wave and are regulated by stretch which is dependent on the activity of the cytoskeleton (Strege *et al.*, 2003a;Strege *et al.*, 2003b;Beyder *et al.*, 2010). In freshly dissociated human intestinal SMCs OB did not inhibit Na^+ currents although a trend but not a significant reduction of the peak current was observed at concentrations of 10 µM (Strege *et al.*, 2004). In contrast, the effect of OB was slightly more potent (IC_{50}=8.8 µM) when Nav1.5 channels were expressed in HEK cells (Strege *et al.*, 2010). We do not know if the cells used in the present study express Nav1.5 channels and future patch-clamp studies are needed to investigate the possible inhibition of OB on Na^+ currents in our experimental conditions. These considerations might be relevant because mibefradil, used in the present work to attenuate $CaCl_2$ induced calcium transients, might also reduce Na^+ currents as it has been demonstrated in HEK cells that express Nav1.5 channels (Strege *et al.*, 2005).

Very little is known about the origin of spasms in the colon. From the patho-physiological point of view a spasm should be considered a pathological sustained contraction often causing pain. A sustained contraction might have a myogenic or neurogenic origin. In the first case voltage gated calcium channels are probably an important source of calcium leading to this contraction. In the second case it is unknown if excitatory motor neurons can fire in excess causing a release of excitatory neurotransmitters (NTs) (possibly ACh and substance P (SP) or NKA) acting on post-junctional receptors. In this case spasm should be due to combination of calcium release from internal stores and influx of calcium through voltage gated channels. In the present paper we show in HCSMCs and in the rat colon that that OB has all those properties: 1-antimuscarinic, 2- voltage gated calcium channel inhibitor and 3-antitachyninergic which in a range of concentrations between 1 and 10 µM can exert their musculotropic antispasmodic properties both in isolated colon cells (human) and in tissue (rat colon).

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DISCUSSION

The aim of this work was to contribute to the current knowledge about the regulatory mechanisms of colonic mechanical activity as well as to investigate the mechanisms of action of several "spasmolytic" mediators capable for inhibiting smooth muscle contractility. Relaxation of colonic smooth muscle can be reached either by activation of the inhibitory pathways or by inhibition of the excitatory ones. In the present study we describe the following mechanisms leading to inhibition of colonic smooth muscle contractility (Figure 1):

- Activation of G protein-coupled receptors (GPCR) related to inhibitory effects on smooth muscle cells (SMCs) (i.e. $P2Y_1$ and EP_2/EP_4 receptors).
- Inhibition of GPCR linked to smooth muscle depolarization and contraction (i.e. muscarinic and tachykinergic receptors) both by Otilonium Bromide (OB) and Hydrogen sulphide (H_2S).
- Blockade of L-type and T-type voltage dependent calcium channels (VDCC) by OB.

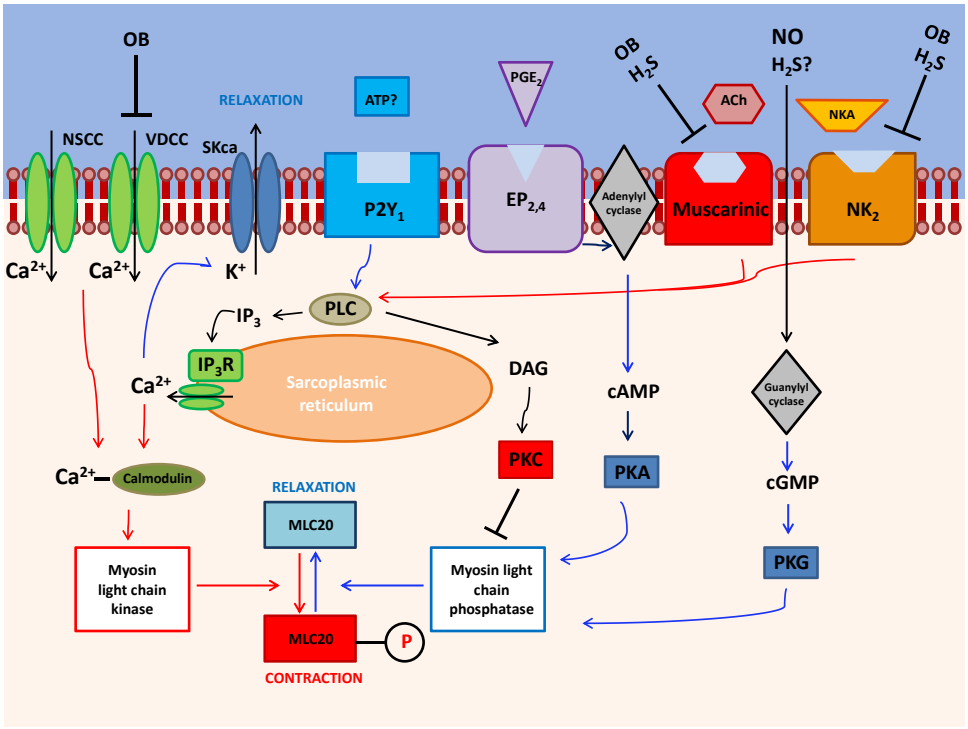


Figure 1. Simplified scheme of the mechanisms regulating contractility in GI SMCs studied in the present work. Mechanisms leading to contraction or relaxation are depicted in red or blue arrows respectively. Ca^{2+} required for excitation-contraction coupling can enter through voltage-dependent calcium channels (VDCC). The open probability of VDCC is enhanced by depolarization caused by excitatory mediators such as acetylcholine (Ach) or tachykinins (TKs) such as neurokinin A (NKA) which evoke the opening of nonselective cation channels (NSCC). Opening of VDCC is decreased by a variety of K^+ channels as small conductance calcium-activated potassium channels (sKca), which can be activated by different inhibitory mediators such as adenosine 5´-triphosphate (ATP) or a related purine activating $P2Y_1$ receptors. Ca^{2+} can also be released from sarcoplasmic reticulum by inositol-1,4,5-trisphosphate receptor operated calcium channels (IP_3R). Inositol 1,4,5-trisphosphate (IP_3) is synthesized by phospholipase C (PLC) in response to agonist binding to inhibitory GPCR such as $P2Y_1$. Intracellular Ca^{2+} binds to calmodulin and activates myosin light chain kinase (MLCK), which phosphorylates 20 kDA light chain of myosin (MLC20) to facilitate cross-bridge formation and contraction. Myosin light chain phosphatase (MLCP) balances the phosphorylation of MLC20 and leads to relaxation. Phenomena that lead to inhibition of MLCP as protein kinase C (PKC) activation increase contraction and enhance Ca^{2+} sensitivity of the contractile apparatus and vice versa. Cyclic nucleotide-dependent pathways decrease Ca^{2+}sensitivity and lead to relaxation: NO binds to guanylyl cyclase (GC) and generates cyclic guanosine monophosphate (cGMP), which activates cGMP-dependent protein kinase (PKG), which can stimulate MLCP and possibly activates potassium channels or close chloride channels on plasma membrane (not shown) leading hyperpolarization. Activation of GPCR such as EP_2 or EP_4 can evoke cAMP production and increase MLCP activity. The spasmolytic drug OB can block Ca^{2+} entry by a direct effect on VDCC or indirectly through inhibition of muscarinic or tachykinergic NK_2 receptors. H_2S may possibly act in synergy with nitric oxide (NO) to activate cGMP related pathways and also inhibit muscarinic and tachykinergic receptors. DAG, diacylglycerol; PGE_2, prostaglandin E_2; PKA, cAMP-dependent protein kinase.

RELAXATION LINKED TO ACTIVATION OF $P2Y_1$ AND EP_2/EP_4 GPCR

PURINE RECEPTORS: $P2Y_1$

Purinergic neurotransmission in the colon: an open debate

At the moment, it is widely accepted that purinergic $P2Y_1$ receptor is critical to the mediation of purinergic relaxation (Burnstock, 2008;Gallego *et al.*, 2006;Gallego *et al.*, 2008c;Gallego *et al.*, 2011;Gallego *et al.*, 2012;Hwang *et al.*, 2012;Gil *et al.*, 2013a). However, a consensus about the identity of the purinergic endogenous transmitter does not exist (Hwang *et al.*, 2011;Goyal, 2011;Jimenez *et al.*, 2014). ATP was proposed in the 70's as the purinergic neurotransmitter (NT) in the gut (Burnstock *et al.*, 1970;Burnstock *et al.*, 1978;Burnstock, 2008;Burnstock, 2014b). Recently, other endogenous purines such as β-NAD and/or its metabolite ADP-ribose have been proposed as possible candidates to act as the endogenous purinergic transmitter (Mutafova-Yambolieva *et al.*, 2007;Hwang *et al.*, 2011;Mutafova-Yambolieva, 2012;Durnin *et al.*, 2012;Durnin *et al.*, 2013;Hwang *et al.*, 2012). Indeed, not only ATP but also β-NAD or even ADP-ribose are considered $P2Y_1$ agonists (Palmer *et al.*, 1998;Abbracchio *et al.*, 2006;Gallego *et al.*, 2006;King & Townsend-Nicholson, 2008;Gustafsson *et al.*, 2011) and all of them are able to cause hyperpolarization and relaxation in the colon (Van Crombruggen & Lefebvre, 2004;Gallego *et al.*, 2006;Mutafova-Yambolieva *et al.*, 2007;Hwang *et al.*, 2011;Gallego *et al.*, 2011;Durnin *et al.*, 2012). The present study has contributed to define the pharmacological profile of the endogenously released purinergic transmitter responsible for the $P2Y_1$ mediated relaxation and fast inhibitory junction potential (IJPf), both in the rat and the human colon and has tested several possible candidates to act as the purinergic NT in the gut. It is important to take into account that a substance should fulfill the following criteria to be identified as a NT (Burnstock, 2014b):

1. The NT should be synthesized and stored in nerve terminals and post-junctionally released after nerve stimulation by a calcium–dependent mechanism.

Many different neurochemical studies have been devoted to the identification and characterization of the purinergic NT involved in the gastrointestinal (GI) relaxation. The release of a NT should be tetrodotoxin (TTX)-sensitive since both electrical field stimulation (EFS) induced and spontaneous inhibitory purinergic neuromuscular transmission are blocked by the voltage-gated sodium channel blocker TTX (Spencer *et al.*, 1998;Gil *et al.*, 2010). By means of HPLC assays, other studies have analyzed the electrically or chemically induced neuronal release of several purines such as ATP or β-NAD and their metabolites including ADP, AMP and adenosine. Such studies have reported that EFS induced release of β-NAD, but not

ATP, occurs in a frequency-dependent manner and is reduced by both TTX and the N-type VDCC blocker ω-conotoxin (ω-CTX) (Mutafova-Yambolieva *et al.*, 2007;Hwang *et al.*, 2011;Durnin *et al.*, 2013). According to these findings, β-NAD and/or their metabolites, but not ATP, may be suitable candidates to act as NT. However, these results should be carefully interpreted. TTX blockade of purine release may not be the best criteria to discard ATP as a NT since high voltages of EFS can directly open VDCC, and thus purine release might in some circumstances of EFS be independent of sodium driven action potentials, that is to say, purinergic neurotransmission may be partially TTX insensitive (Pluja *et al.*, 1999). Furthermore, it has been demonstrated that ω-CTX may not completely block neither the IJP in the rat colon (Borderies *et al.*, 1996) nor NT release from postganglionic sympathetic nerves in guinea-pig vas deferens (Smith & Cunnane, 1998), suggesting that other calcium channel such as P, Q or R type can participate in NT release. Finally, it might be also possible that EFS induced release of purines from nerve terminals might overlap with partial release of non-neural origin giving at the end confusing results.

2. The NT should act on a defined receptor and an antagonist should be able to block the response to nerve stimulation.

Our results agree with previous studies that demonstrated that the EFS-induced non-nitrergic relaxation is fully antagonized by MRS2500 (Gallego *et al.*, 2011) and also partially by apamin in the human colon (Gallego *et al.*, 2006), suggesting that the purinergic component of the colonic relaxation is mediated by $P2Y_1$ receptors and sKca potassium channels. Other purinergic receptors as $P2Y_{11}$, (which might be absent in rodents (Abbracchio *et al.*, 2006)), have been proposed to be involved in purinergic relaxation (King & Townsend-Nicholson, 2008). However, we observed no effect of the $P2Y_{11}$ antagonist NF157 10 μM on the EFS induced purinergic mechanical relaxation in human colonic tissue. Accordingly, our results confirm that $P2Y_1$ is the receptor involved in the purinergic relaxation and therefore the effect induced by endogenous NT/agonist of such receptor should be blocked by the antagonist MRS2500.

3. Exogenous addition should mimic EFS-induced endogenous responses.

αβ-meATP, a synthetic analogue of ATP, was the purine that better fulfilled the pharmacological criteria of the endogenously released purinergic transmitter both in the human and rat colon. Indeed, αβ-meATP showed a similar pharmacological profile as the $P2Y_1$ receptor agonist ADPβS. The main results obtained are summarized in the following table:

Table 1. EC_{50} values obtained for the purines tested in rat and human colon in control conditions and after incubation with MRS2500 or apamin (data from Chapter 1).

	RAT			HUMAN		
	Control	**MRS2500 1 µM**	**Apamin 1 µM**	**Control**	**MRS2500 1 µM**	**Apamin 1 µM**
α,β-meATP	2.7 µM	ND***	ND***	4.4 µM	ND***	ND***
ATP	27.9 µM	49.8 µM	86.2 µM	123.7 µM	370.9 µM	91.0 mM**
ADP	72.5 µM	59.6 µM	105.2 µM	272.8 µM	323.8 µM	1.2 mM
β-NAD	74.1 µM	48.4 µM	69.8 µM	5.8 mM	5.1 mM	6.4 mM
ADP-ribose	34.9 µM	35.4 µM	37.3 µM	NT		
ADPβS	NT			1.1 µM	9.3 µM ***	2.6 mM***

ND: not determined (the agonist is completely blocked); NT: not tested; * P<0.05; **P<0.01; ***P<0.001 (Two-Way ANOVA compared to control).

In our study, any of the endogenously synthesized purines tested exactly mimicked the endogenously released NT. Only ATP partially fulfilled the pharmacology of the endogenous purine since its inhibitory effects were reduced by apamin. A striking point is how the classical P2X agonist αβ-meATP was able to exactly mimic the pharmacological profile of the endogenous purine. Table 2 collects some of the main effects of αβ-meATP reported in the literature:

Table 2. Reported effects of αβ-meATP on smooth muscle in different species and areas of the GI tract as well as the receptors involved according to the antagonists tested or the response observed.

Effect	Species	Tissue	Antagonist	Receptors	Reference
Hyperpolarization and relaxation	Guinea-pig	Colon	PPADS 30 µM Suramin 100 µM Apamin 0.1 µM	P2 Linked to sKca	Zagorodnyuk *et al.*, 1996
Hyperpolarization Reduction of IJPf	Human	Jejunum		P2	Xue *et al.*, 1999
Relaxation	Rat	Ileum	PPADS 30 µM Apamin (IC_{50}=1.5 nM)	P2 Linked to sKca	Storr *et al.*, 2000
Relaxation	Rat	Pylorus	PPADS 30 µM	P2	Ishiguchi *et al.*, 2000
Relaxation[1] Contraction	Mouse	Different areas		Putative P2Y	Giaroni *et al.*, 2002
Relaxation[1]	Mouse	Jejunum	Evans blue 10 µM NF279 1 µM	P2X	De Man *et al.*, 2003
Depolarizaton and contraction	Dog	colon		$P2X_2$ $P2X_3$ $P2X_4$	Lee *et al.*, 2005
Relaxation	Rat	Distal colon	Reactive blue 2 (>1 µM) PPADS (>1 µM) Suramin (>3 µM) MRS2179 (>3 µM)	P2 $P2Y_1$	Van Crombruggen *et al.*, 2007
Fast relaxation	Guinea-pig	Colon	A317491	P2X	King & Townsend-Nicholson, 2008
Slow relaxation	Guinea-pig	Colon	Reactive red 100 µM	$P2Y_{11}$	King & Townsend-Nicholson, 2008
Calcium transient	Human astrocytoma cell line stably expressing $P2Y_{11}$		Reactive red (IC_{50}= 72.4 µM)	$P2Y_{11}$	King & Townsend-Nicholson, 2008
Relaxation	human	Colon	NF2179 10 µM MRS2179 10 µM	P2X $P2Y_1$	Auli *et al.*, 2008

PPADS has been widely used as a P2X antagonist and a blockade of αβ-meATP effects has been reported in different studies (Zagorodnyuk *et al.*, 1996;Storr *et al.*, 2000;Ishiguchi *et al.*, 2000). Nevertheless, it should be taken into account that PPADS is not currently considered a selective inhibitor since antagonism on P2Y receptors has been reported (Abbracchio *et al.*, 2006;Alexander *et al.*, 2011), and therefore it may be mistaken to conclude that αβ-meATP is acting exclusively as a P2X agonist based on those results. Moreover, other studies have revealed a possible activation of $P2Y_1$ receptors/sKca channels (Zagorodnyuk *et al.*, 1996;Storr *et al.*, 2000;Auli *et al.*, 2008;King & Townsend-Nicholson, 2008). In the present study we have also reported a blockade of αβ-meATP inhibitory effects by the P2X antagonists NF023 and iso-PPADS in the rat colon. However, it is important to remark that both NF023 and iso-PPADS partially inhibited αβ-meATP induced relaxation at the same concentration (10 μM) at which the $P2Y_1$-mediated relaxation and the IJPf were also partially antagonized.

It is important to bear in mind that when purinergic relaxation is studied by means of exogenously added drugs to an organ bath, it is easy to activate purinergic receptors located "extrajunctionally" that may not be activated by focally released purines from enteric neurons which activate receptors specifically located at the neuromuscular junction. For instance, it has been reported that GI relaxation can be mediated by different P2Y receptors located either in neurons or in smooth muscle (Giaroni *et al.*, 2002). Accordingly, the purinergic substances tested that do not fulfil the pharmacological criteria of the endogenous purine in the present work (and also the products of their metabolism) may activate other P2Y receptors different from $P2Y_1$ which may directly or indirectly participate in smooth muscle relaxation (Abbracchio *et al.*, 2006). Indeed, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{14}$ receptors have been localized in the human colon and are expressed in different cell types as enteric neurons, SMCs, interstitial cells of Cajal (ICCs), platelet derived growth factor receptor α-positive cells (PDGFRα+ cells) and glial cells (Van Crombruggen & Lefebvre, 2004;Gallego *et al.*, 2006;Giaroni *et al.*, 2002;Giaroni *et al.*, 2006;Monaghan *et al.*, 2006;King & Townsend-Nicholson, 2008;Kurahashi *et al.*, 2011;Van Nassauw *et al.*, 2006). A striking point about our study is the capability of exogenously added αβ-meATP to perfectly mimic the endogenous purine both in human and rat colon despite that numerous purinergic receptors can also be activated.

4. The released NT should be inactivated by ectoenzymes or by an uptake mechanism.

It is well known that endogenously produced purines are quickly metabolized by ectonucleotidases. Accordingly, the term "ATP or a related purine" is commonly used in the literature since ecto-nucleotidase metabolism quickly converts ATP into ADP or adenosine, molecules that might also be bioactive (Duarte-Araujo *et al.*, 2009). In the present study we

show that the exogenous addition of αβ-meATP, the stable analogue of ATP, perfectly mimics the pharmacological profile of the endogenous purine. Accordingly, we tested the effects of ATP in the presence of the ectonucleotidase blocker ARL67156 in the rat colon. ATP inhibitory effects were not modified in the presence of ARL67156, suggesting that ATP may not be the purinergic transmitter. However, previous studies have suggested that ARL67156 potentiates UTP but not ATP or ADP effects, suggesting an unequal efficacy on the different subtypes of ecto-nucleotidases and possibly an inefficient blockade of ATP metabolism (Van Crombruggen *et al.*, 2007). Therefore, more selective ectonucleotidase inhibitors are needed in order to discard or confirm the activation of $P2Y_1$ receptor by the intact ATP molecule.

Taken together, our findings demonstrate that the synthetic ATP analogue αβ-meATP, classically considered as a P2X agonist, is the purinergic agonist tested that better mimics the endogenous transmitter both in the rat and human colon. However, our study has not solved the discrepancies about the identity of the purinergic NT. It may be possible that a wide variety of purines including ATP, β*-NAD or even their metabolites might be participating in purinergic inhibitory neuromuscular responses. Therefore, multiple purines released by enteric neurons and bioactive compounds junctionally and transiently created might be targeting multiple cell targets in the neuroeffector syncytium in smooth muscle. Further research is needed to clarify the nature of the purine(s) involved in the colonic inhibitory responses and how all these substances engage in co-regulation of effector systems.*

EP_2 AND EP_4 RECEPTORS

Prostaglandin E_2 regulates GI contractility through EP_2 and EP_4 receptors

Prostaglandin E_2 (PGE_2) is involved in many different physiological processes, including the regulation of GI motility (Dey *et al.*, 2006). Four different target receptors have been associated to PGE_2: EP_1, EP_2, EP_3 and EP_4 (Narumiya & FitzGerald, 2001;Sugimoto & Narumiya, 2007). According to their effects on the smooth muscle, these receptors have been classified as relaxant EP_2 and EP_4 receptors and contractile EP_1 and EP_3 receptors (Narumiya *et al.*, 1999). Accordingly, it has been reported that PGE_2 is able to elicit both a contractile and a relaxant effect in several areas of the GI tract and in different species (Bennett *et al.*, 1968a;Bennett *et al.*, 1968b;Bennett *et al.*, 1981;Botella *et al.*, 1995;Dey *et al.*, 2006).

Similarly to previous studies performed in human aortic SMCs (Pantazaka *et al.*, 2013) and in mouse airway smooth muscle (Srivastava *et al.*, 2013), in the present study we demonstrate that both EP_2 and EP_4 receptors participate in the inhibitory effect of PGE_2. The

intracellular pathway linked to activation of either EP_2 or EP_4 receptors is related to cAMP increase and smooth muscle relaxation (Figure 1) (Narumiya & FitzGerald, 2001). Our study has demonstrated that the inhibition of spontaneous contractions or hyperpolarization induced by PGE_2 is still produced after incubating with the EP_2 antagonist PF-04418948 or the EP_4 antagonist L-161,982, and the EC_{50} is similar in both cases, suggesting that PGE_2 is able to cause similar inhibitory responses through either EP_2 or EP_4 receptors. Accordingly, a combination of both EP_2 and EP_4 antagonists is needed to block PGE_2 effect in WT animals whereas the EP_4 antagonist L-161,982 alone totally blocks PGE_2 effects in EP_2-KO mice. An interesting point about our study is that the blockade of one receptor does not prevent the other one to mediate an effective relaxation, suggesting that both EP_2 and EP_4 GPCR can independently lead to inhibition of contractility in murine circular colonic smooth muscle and thus both of them are putative targets to treat colonic dismotility.

Purinergic or nitrergic components of the inhibitory neurotransmission are not involved in PGE_2 inhibitory effects

Both purinergic and nitrergic NTs released by enteric inhibitory motor neurons have a crucial role in the regulation of GI motility (Grasa *et al.*, 2009;Gallego *et al.*, 2008b;Gallego *et al.*, 2011;Mane *et al.*, 2014;Jimenez *et al.*, 2014;Gallego *et al.*, 2012). We have demonstrated that activation of both the $P2Y_1$ purine receptor and EP_2/EP_4 prostanoid receptors lead to hyperpolarization and relaxation of colonic smooth muscle (Figure 1). Prior studies have reported the involvement of prejunctional EP receptors in GI motility regulation (Fairbrother *et al.*, 2011) as well as the interaction of PGE_2 with both the purinergic and nitrergic pathways (Hristovska *et al.*, 2007;Traves *et al.*, 2013). Taking into account all these findings, we found rellevant to test the effects of TTX, L-NNA and MRS2500 on PGE_2 mediated relaxation as well as the actions of PGE_2 on the purinergic and nitrergic components of the neurotransmission. Our results show that PGE_2 induced relaxation is TTX-insensitive, and therefore this mediator is acting directly on smooth muscle. Moreover, since previous incubation with L-NNA or MRS2500 did not modify the inhibitory effects of PGE_2 on mechanical activity, and PGE_2 did not alter nitrergic or purinergic components of the neurotransmission, a possible interaction with purinergic or nitrergic inhibitory pathways was discarded. Taken together, these findings correlate with a direct effect of PGE_2 on EP_2 and EP_4 receptors located post-junctionally on colonic smooth muscle.

Constitutively produced prostaglandins might contribute to the maintenance of the inhibitory tone

It is widely accepted that constitutive production of NO regulates smooth muscle tone and contractility in the GI tract. Accordingly, depolarization and increase of contractility is observed after blocking NO synthases with L-NNA or GC with ODQ (Keef *et al.*, 1997;Gil *et al.*, 2010;Gil *et al.*, 2012). However, other substances may possibly be involved in the maintenance of the basal inhibitory tone in GI smooth muscle. Both cyclooxygenase-1 (COX-1) and COX-2 enzymes, responsible for PG synthesis (including PGE_2) are constitutively expressed in mouse, rabbit or human GI tract (Zimmermann *et al.*, 1998;Cosme *et al.*, 2000;Roberts *et al.*, 2001;Porcher *et al.*, 2004;Fornai *et al.*, 2005;Bernardini *et al.*, 2006). A significant increase of spontaneous contractility has been reported in murine colon after blockade of COX enzymes (Porcher *et al.*, 2004), suggesting that constitutively produced prostaglandins (PGs), probably including PGE_2, might contribute to maintain the colonic SMCs hyperpolarised and inhibited. In the same line with this hypothesis, our study reports that both EP_2 and EP_4 receptors are involved in the tonic inhibitory control of smooth muscle excitability since the blockade of each one leads to smooth muscle depolarization and increase of contractility in an additive manner.

Both EP_2 and EP_4 receptors independently mediate PGE_2 induced smooth muscle hyperpolarization and inhibition of contractility in mouse colon. PGE_2 relaxant effect is not due to prejunctional activation of EP receptors or to interaction with purineric or nitrergic components of the inhibitory neurotransmission. Our findings also suggest that constitutively produced PGs, probably PGE_2, contribute to the maintenance of a smooth muscle inhibitory tone by activation of both EP_2 and EP_4 receptors.

HYDROGEN SULPHIDE AS A PUTATIVE GASOUS MEDIATOR IN THE HUMAN COLON

The enzymes responsible for H_2S production cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) are expressed by different cell types in the murine colon (Linden *et al.*, 2008;Hennig & Diener, 2009;Martin *et al.*, 2010;Gil *et al.*, 2011;Liu *et al.*, 2013) and by ICCs and neurons in the human colon (Schicho *et al.*, 2006). Moreover, the endogenous production of H_2S has been demonstrated both in the mouse (Linden *et al.*, 2008) and the rat colon (Gil *et al.*, 2011). Since H_2S is endogenously produced and has demonstrated to cause concentration-dependent relaxation on smooth muscle contractility in different species and areas of the GI tract (Hosoki *et al.*, 1997;Teague *et al.*, 2002;Gallego *et al.*, 2008a;Jimenez, 2010;Dhaese *et al.*, 2010;Gil *et al.*, 2011), its putative role as an inhibitory NT has been widely hypothesized. At the moment, the physiological relevance and the potential therapeutic use of H_2S are still under debate, and neither the target nor the mechanism of action linked to H_2S have been clearly defined (Jimenez, 2010). Several findings reported in the present study support the role for H_2S in the regulatory mechanisms of GI motility in the human colon:

- The H_2S donor NaHS efficiently inhibited spontaneous contractility in the presence of TTX, suggesting a direct effect on smooth muscle. Moreover, the GC blocker ODQ significantly reduced H_2S relaxant effect, suggesting a possible synergy with NO.
- A combination of CSE inhibitor D,L-propargylglycine (PAG) and the CBS inhibitor aminooxyacetic acid (AOAA) depolarized smooth muscle resting membrane potential (RMP) and caused a transient tone increase, suggesting an equal implication for both enzymes in the tonically synthesized H_2S in the human colon. These results partially correlate to previously reported findings (Gil *et al.*, 2011), and suggest that endogenously produced H_2S contributes to the maintenance of an inhibitory tone in the human colon.
- NaHS reversibly blocked both the electrically and chemically induced cholinergic and tachykinergic contractions, suggesting an inhibitory effect of H_2S on excitatory pathways in the human colon.

Selectivity of the inhibitors of H_2S producing enzymes in doubt

The CSE inhibitor PAG and the CBS inhibitors AOAA (both used in the present work) and hydroxylamine (HA), have been widely used in experiments performed with tissue homogenates and isolated cells (Hosoki *et al.*, 1997;Stipanuk & Beck, 1982;Szabo, 2007;Linden *et al.*, 2010). However, the selectivity of these compounds is currently in doubt due to the fact that undesirable effects on other enzymes or receptors have been observed (John & Charteris,

1978;Teague *et al.*, 2002;Szabo, 2007;Jimenez, 2010;Whiteman *et al.*, 2011). In the present work, HA caused a TTX-insensitive concentration-dependent inhibition of mechanical activity of human colonic strips antagonized by the GC blocker ODQ, confirming the previously reported lack of selectivity and NO-like effects of HA (Iversen *et al.*, 1994;Correia *et al.*, 2000;Gil *et al.*, 2011). Accordingly, although our results suggest that constitutively produced H_2S contributes to the maintenance of an inhibitory tone in the human colon, it is important to bear in mind that such findings may also be related to non-selective effects of PAG or AOAA. More selective blockers of both CSE and CBS are required in order to better characterize the role of H_2S in the GI tract.

Mechanism of action of H_2S

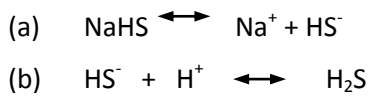
The signalling pathways of H_2S may be complex and multiple. Different target proteins and different mechanisms of action have been postulated to explain H_2S effects, including activation of ATP-sensitive potassium channels (K_{ATP}) and sKca channels (Zhao *et al.*, 2001;Cheng *et al.*, 2004;Gallego *et al.*, 2008a;Nagao *et al.*, 2012;Linden, 2014), T-type calcium channels (Matsunami *et al.*, 2009), cAMP-dependent pathways (Kimura, 2000), MLCP (Dhaese & Lefebvre, 2009;Nagao *et al.*, 2012), TRPV1 channels (Krueger *et al.*, 2010), sodium channels (Strege *et al.*, 2011), synergy with NO (Hosoki *et al.*, 1997;Teague *et al.*, 2002), sulfhydration (Mustafa *et al.*, 2009) or even inhibition of cholinergic responses (Teague *et al.*, 2002;Kasparek *et al.*, 2012;Gil *et al.*, 2013b;Castro-Piedras & Perez-Zoghbi, 2013).The results shown in the current work suggest a possible synergistic effect of H_2S with NO and support the previously hypothesized anticholinergic effects of H_2S. Moreover, a possible inhibitory effect of H_2S on the tachykinergic pathway has been demonstrated too (Figure 1). Further investigation is needed in order to define the target of H_2S inhibitory effects.

High concentration of H_2S in the organ bath, unknown effective concentration inside the tissue

The IC_{50} values corresponding to the inhibitory effect of H_2S on spontaneous contractility (329.2 μM) or those reported in table 3 are quite high when compared for instance to other compounds capable for eliciting an effective inhibition of spontaneous contractility in the human colon, as the stable purine analogues α,β-meATP or ADPβS (IC_{50} values are 4.4 μM and 1.1 μM respectively). However, there are different issues that should be taken into account.

The reported physiological concentrations of H_2S in rat blood, human blood and rat brain are in a range between 50-100 μM (Zhao *et al.*, 2001;Teague *et al.*, 2002). In 2010,

Linden stated: "it becomes important to determine what actions of H_2S/HS^- observed in vitro studies reflect toxicity to tissue versus physiologically relevant signaling of tissue activity" (Linden *et al.*, 2010). It is important to note that although high concentrations of H_2S were used in our studies, the effect of H_2S was recovered after washout, discarding an irreversible impairment of muscle function caused by toxicity. Another interesting point is that due to the slow diffusion of H_2S into tissues (even if the pieces are relatively small), the final concentration inside the strip may be much lower or even trivial (Linden *et al.*, 2010). NaHS is a H_2S sodium salt that dissociates to form sodium cation (Na^+) and hydrosulphide anion (HS^-) in solution (a). Under physiological conditions, HS^- anions associate with protons (H^+) and produce H_2S (a) since the dissociation of HS^- to H^+ and sulphide (S^{2-}) is negligible.



(a) $NaHS \leftrightarrow Na^+ + HS^-$

(b) $HS^- + H^+ \leftrightarrow H_2S$

It is important to remark that under common experimental conditions, the undissociated H_2S constitutes only the 18.5 % (Dombkowski *et al.*, 2004;Linden *et al.*, 2010). Moreover, it has not yet been determined which form of H_2S (H_2S, HS^- or S^{2-} or the mix) is the active one (Kimura, 2010). It is important to take into account other phenomena that might reduce the amount of effective molecules that arrive to the target inside the tissue, for instance evaporation (Jimenez, 2010;Kimura *et al.*, 2006), protein binding (Ishigami *et al.*, 2009;Kimura, 2010) or degradation (Linden *et al.*, 2012). Accordingly, it seems difficult to exactly determine the real concentration of H_2S during the experiments. Therefore, although the effective concentrations of NaHS used in our study are in the rage of mM, it seems possible that the real concentration of the active form of H_2S inside the tissue is much lower.

According to the previously reported findings and the results presented in the present work, H_2S fulfills, at least partially, the criteria to be considered a gasomediator in the colon. It may be possible that H_2S is produced and released in a tonic manner in the human colon and exerts an inhibitory control on SMC membrane potential as well as on cholinergic and tachykinergic neuromuscular transmission. Further investigation is needed in order to define the real physiological relevance of H_2S in the GI tract.

RELAXATION LINKED TO INHIBITION OF MUSCARINIC AND TACHYKINERGIC GPCR

In the present study we demonstrate that cholinergic and tachykinergic receptors can be inhibited both by OB and the H_2S donor NaHS, although OB is much more potent than H_2S. The following table summarizes the IC_{50} values:

Table 3. Comparison between the IC_{50} values obtained for NaHS and OB inhibitory effect on electrically or chemically induced cholinergic and tachykinergic contractions and for OB inhibitory effect on the intracellular calcium increase elicited either by carbachol and NKA.

	Preparation/ experimental procedure	Anticholinergic		Antitachykinergic	
		EFS-induced contraction[1]	Carbachol-induced contraction (ΔAUC)[1] or $\Delta[Ca^{2+}]_i$ [1]	EFS-induced contraction[2]	NKA-induced contraction (ΔAUC) [2] or $\Delta[Ca^{2+}]_i$ [3]
H_2S	Human colonic strips/ organ bath	2 mM	1.9 mM	5.7 mM	1.7 mM
OB	Rat colonic strips/ organ bath	7.3 μM	13 μM	84.4 μM	56.1 μM
	Isolated HCSMCs/ Calcium imaging	---	8.2 μM	----	17 μM

ΔAUC, Area under curve increase; $\Delta[Ca^{2+}]_i$, Intracellular calcium increase; HCSMCs, human cultured colonic smooth muscle cells; [1] Blocked by atropine; [2] Blocked by the NK_2 antagonist GR159897 (human); [3] blocked by the NK_2 antagonist nepadutant. EFS, electrical field stimulation; Human colon: EFS to elicit cholinergic response 0.4 ms, 50 Hz, 50 V, 1 s (in the presence of MRS2500 1 μM and L-NNA 1 mM); EFS to elicit tachykinergic response 0.4 ms, 50 Hz, 50 V, 10 s (in the presence of MRS2500 1 μM and L-NNA 1 mM and atropine 10 μM). Rat colon: EFS to elicit cholinergic response 0.3 ms, 50 Hz, 50 V, 1 s (in the presence of MRS2500 1 μM and L-NNA 1 mM).

MUSCARINIC RECEPTORS

ACh is considered the main excitatory NT in the gut (Maggi *et al.*, 1997;Lecci *et al.*, 2002). In the present study we show that an atropine-sensitive contraction can be electrically and chemically (by exogenous addition of carbachol) evoked both in human and rat muscle strips (EFS duration: 1 s). Accordingly, infusion of carbachol evokes atropine-sensitive intracellular calcium rise in human cultured colonic smooth muscle cells (HCSMCs). In addition, a cholinergic excitatory junction potential (EJP) can be evoked in the rat colon (Gil *et al.*, 2013b). It is important to use "non-nitrergic, non-purinegic" conditions to study neural mediated cholinergic pathways otherwise the presence of a rebound contraction at the end of an inhibitory stimulus (off-contraction) (Auli *et al.*, 2008) overlap with the cholinergic contraction and therefore results are often difficult to interpret.

Our results demonstrate that OB inhibits cholinergic responses and agree with the previously reported antimuscarinic effects of OB (Evangelista *et al.*, 1998;Santicioli *et al.*, 1999). Moreover, our study shows that OB is able to inhibit L-type VDCC too (Figure 1) (Evangelista *et al.*, 1998;Martin *et al.*, 2004;Strege *et al.*, 2010;Strege *et al.*, 2004;Gallego *et al.*, 2010). It is well known that excitatory mediators such as ACh or TKs depolarize SMC membrane potential and consequently evoke L-type calcium channels opening (Sanders,

2000;Sanders, 2008). Therefore, in the present study we demonstrate the antimuscarinic (and also the antitachykinergic) effect of OB in the presence of nifedipine in order to ensure a real effect of the drug on the receptor (and not on the channel) and to find out the relative contribution of each mechanism of action of OB. Here we also demonstrate that the H_2S donor NaHS blocks both the EFS and carbachol induced cholinergic contractions in the human colon. Our results support the antimuscarinic effect of H_2S reported in previously published studies in animals (Teague *et al.*, 2002;Kasparek *et al.*, 2012;Castro-Piedras & Perez-Zoghbi, 2013;Gil *et al.*, 2013b) and demonstrate similar results in human colonic tissue.

NK_2 RECEPTORS

Together with ACh, TKs are excitatory NTs in the GI tract (Shimizu *et al.*, 2008). Several studies suggest that in the human sigmoid colon tachykinergic responses involve mainly NK_2 receptors, suggesting a relevant role for NKA (Cao *et al.*, 2000;Auli *et al.*, 2008). It has been previously reported that in the presence of blockers of inhibitory neurotransmission, a single pulse of EFS is able to induce an atropine-sensitive cholinergic contraction. Nevertheless, trains of pulses and a higher stimulus are needed to induce a tachykinergic response (Maggi *et al.*, 1997). In the same line with such studies, in the present work we demonstrate that in "non-nitrergic, non purinergic and non-muscarinic conditions", when the duration of EFS is increased from 1 to 10 s, a prominent non-cholinergic contraction can be recorded (see Table 3). Under these experimental conditions, both EFS (10 s) and exogenously added NKA, evoked a NK_2 antagonist-sensitive contraction in the human colon. Moreover, infusion of NKA evoked calcium transients in HCSMCs which are NK_2 antagonist-sensitive too.

The present study supports the reported antitachykinergic properties of OB (Santicioli *et al.*, 1999) due to the fact that OB was able to inhibit NKA induced calcium transients in a similar way to the NK_2 receptor antagonist nepadutant. OB also blocked NKA induced contractions in rat colon strips, however, the effects of NKA were not antagonized by the NK_2 antagonist nepadutant (not shown). Similarly to OB, the H_2S donor NaHS blocked both the electrically and chemically induced tachykinergic contractions in the human colon (Figure 1).

Non-purinergic, non-nitrergic conditions are necessary to inhibit neural mediated inhibitory responses. Under these experimental conditions short trains (1 s) of EFS induce an atropine-sensitive cholinergic response whereas in the presence of atropine, long trains (10 s) of EFS induce a tachykinergic response. This experimental protocol allows the proper characterization of neural mediated excitatory inputs on SMCs. We propose that these

experimental conditions are suitable to test drugs with potential spasmolytic actions due to decrease of excitatory inputs to smooth muscle.

Both H_2S and the commonly used spasmolytic drug OB are able to block the electrically and chemically evoked muscarinic and tachykinergic responses in the colon. However, the potency of OB is considerably higher when compared to H_2S.

RELAXATION LINKED TO BLOCKADE OF L-TYPE AND T-TYPE VDCC

Both L-type and T-type VDCC have been identified in SMCs and ICCs in the GI tract (Gibbons *et al.*, 2009;Beyder & Farrugia, 2012). L-type calcium channels are the main responsible for calcium entry into SMC that triggers contraction. Nevertheless, T-type calcium channels also participate in GI motility regulation (Beyder & Farrugia, 2012) and contribute to the upstroke of the pacemaker potential (Gibbons *et al.*, 2009). In the present work we demonstrate the inhibitory effects of the spasmolytic drug OB on both L-type and T-type calcium channels.

OTILONIUM BROMIDE

OB, a commonly used spasmolytic drug, has beneficial effects in the treatment of gut motor disorders related to irritable bowel syndrome (IBS) (Battaglia *et al.*, 1998;Chang *et al.*, 2011;Clave *et al.*, 2011). OB is a safe drug, devoid of important side effects (Forte *et al.*, 2012) and is probably acting directly on intestinal SMCs to reduce contractility (Boeckxstaens *et al.*, 2014). Four pharmacological properties have been attributed to OB effects, including VDCC blocker (including L and T-type) (Evangelista *et al.*, 1998;Martin *et al.*, 2004;Strege *et al.*, 2004;Strege *et al.*, 2010;Gallego *et al.*, 2010), antimuscarinic (Lindqvist *et al.*, 2002) and antitachykinergic (Cipriani *et al.*, 2011) (the two latter properties of OB have been previously discussed above). It might be difficult to define the relative contribution of each property to the final effect of OB since these four mechanisms are linked: activation of excitatory membrane receptors can lead to release of calcium from the sarcoplasmic reticulum that in turn depolarizes de membrane potential causing L-type VDCC opening (Sanders *et al.*, 2012). In the present work we have proposed the study of intracellular calcium transients in isolated HCSMCs as a model that allows the differentiation between the four mechanisms of action related to OB musculotropic spasmolytic effects.

Besides from the previously discussed antimuscarinic and antitachykinergic effects, in the present work we demonstrate that OB blocks the L-type VDCC mediated contractile

responses in rat colonic strips (EC_{50}= 9.44 μM) and BayK8644 and KCl evoked intracellular calcium increase in isolated HCSMCs (EC_{50}= 3.35 μM and 5.1 μM respectively) (Figure 1). Morevover, OB inhibits T-type calcium channels in HCSMCs (EC_{50}=18 μM) but with a lower potency when compared to L-type calcium channels. An interesting point is that OB has similar pharmacological properties in mechanical/electrophysiological studies performed in rat colonic strips and in isolated HCSMCs (calcium imaging) (see Table 3). Finally, it is important to bear in mind that the other inhibitory mediators studied in the present work (purines, H_2S and PGE_2) will also reduce the opening probability of VDCC due to their capability of hyperpolarizing the smooth muscle membrane potential (Table 4).

OB blocks L-type and T-type VDCC and muscarinic and TK receptors. In the present study we have developed a pharmacological approach to elucidate the relative contribution of each property of OB to its spasmolytic effect. The four different mechanisms of action of OB acting in synergy might explain its effectiveness in the treatment of colonic motor disorders.

COMPARISON BETWEEN ATP (OR A RELATED PURINE), PGE_2 AND H_2S AS POTENTIAL MEDIATORS OF COLONIC MOTILITY

In the present work we have investigated the inhibitory effects on colonic motility as well as the pharmacology and the pathways involved in ATP (or a related purine), PGE_2 and H_2S mediated responses. A summary is reported in the following table:

Table 4. Comparative view between the inhibitory effects mediated by ATP or a related purine, PGE_2 and H_2S in the colon.

	ATP (or related purine)	**PGE_2**	**H_2S**
Pathways / pharmacology			
Synthesis	Mitochondria	COX-1 and COX-2	CBS, CSE
Possible NT	ATP/ADP, β-NAD/ADP-ribose (mimicked by α,β-meATP)	PGE_2 (and other PGs)	H_2S
Receptor/Pathway	$P2Y_1$ / SK_{Ca} (apamin)	EP_2, EP_4/ (cAMP)	Unknown
EC_{50} (inhibition of contractility)	α,β-meATP: 4.4 μM (human), 2.7 μM (rat)	10.6 nM (WT mice) 23.6 nM (EP_2-KO mice)	329.2 μM (human)
Antagonists[1] or synthesis inhibitors[2]	MRS2500[1] ($P2Y_1$)	PF-04418948[1] (EP_2), L-161,982[1] (EP_4)	AOAA[2](CBS), PAG[2](CSE)
Effect of agonists / donors			
Effect on RMP	Hyperpolarization	Hyperpolarization	Hyperpolarization
Effects on contractility	Relaxation	Relaxation	Relaxation
Other effects			Inhibition of cholinergic and tachykinergic contractions
Effect of antagonists/ Synthesis Inhibitors[3]			
	Blockade of EFS induced IJPf and purinergic relaxation	Depolarization of RMP and increase of contractility	Depolarization of RMP and transient tone increase

CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; β-NAD, β-nicotinamide adenine dinucleotide; ADP-ribose, adenosine 5'-diphosphate-ribose; cAMP, cyclic adenosine monophosphate; H_2S, hydrogen sulphide; sK_{Ca}, small conductance calcium-activated potassium channels; AOAA, aminooxyacetic acid; PAG, D,L-propargylglycine; IJPf, fast inhibitory junction potential; RMP, resting membrane potential; COX, cyclooxygenase; PGE_2, prostaglandin E_2; PGs, prostaglandins. NT not tested in the present work

[3] tested with MRS2500 for purines, PF-04418948 and L-161,982 for PGE_2 and a combination of PAG (2 mM) and AOAA (1 mM) for H_2S.

This comparative table shows some similarities between the three mediators. The purinergic transmitter, PGE_2 and H_2S are all endogenously synthesized molecules capable of producing inhibition of spontaneous contractions and hyperpolarization. Moreover, the blockade of the target receptors (EP_2/EP_4) of PGE_2 as well as the inhibition of endogenous synthesis of H_2S depolarizes the RMP, suggesting their participation in the maintenance of the inhibitory tone in the colon.

POSSIBLE THERAPEUTIC APPLICATIONS OF PURINES, PGE_2 AND H_2S RELATED PATHWAYS: A POSSIBLE ROLE IN THE TREATMENT OF GUT MOTILITY DISORDERS

IBS is a complex chronic functional bowel disease associated to disorders in defecation (constipation, diarrhoea or mixed/alternated) (Longstreth *et al.*, 2006;Boeckxstaens *et al.*, 2014). The pathophysiology of IBS is poorly understood, and therefore the therapy is sometimes a symptom-based approach (Drossman *et al.*, 2002;Rychter *et al.*, 2014).

There are three main classes of spasmolytics or smooth muscle relaxants commonly used in the treatment of IBS: calcium channel antagonists, direct smooth muscle relaxants and anticholinergic/antimuscarinic agents (Boeckxstaens *et al.*, 2014). In the present work, four kinds of molecules with spasmolytic activity capable for reducing the colonic contractility have been tested: purines, PGE_2, H_2S and OB, being the latter the only compound currently used as a spasmolytic drug in the treatment of dismotility related to gut inflammation (Forte *et al.*, 2012). In the present study we demonstrate four mechanisms of action of OB that when acting in synergy might explain its spasmolytic properties: L-type and T-type VDCC blocker, antimuscarinic and antitackykinergic. Similarly, prior studies have investigated the mechanism of action of the antimuscarinic agent hyoscine butylbromide (Krueger *et al.*, 2013) and have demonstrated its capability of concentration-dependently reduce the electrically and chemically induced muscarinic contractions in the human intestine. Moreover, it has also been reported that the NK_2 antagonist idobutant inhibits NK_2 mediated contractions in the human colon (Santicioli *et al.*, 2013). These three compounds probably share similarities with H_2S since, in the present work, we demonstrated antimuscarinic and antitachykinergic effects in the human colon. Finally, due to the fact that the frequency of propulsive contractions is often increased in motility disorders associated to IBS (Gonzalez & Sarna, 2001;Sethi & Sarna, 1991; Sarna 2010) and propulsive colonic motility patterns may be RMP dependent (Huizinga *et al.*, 2011;Gil *et al.*, 2013b), hyperpolarizing molecules such as NO, H_2S, PGE_2 or purines might be considered as potential pharmacological tools to treat hypermotility.

The following table shows possible pros and cons of the potential therapeutic use of the compounds tested in the present study:

Table 5. Pros and cons of the therapeutic use of OB, H_2S, $P2Y_1$ agonists and PGE_2 in GI motility disorders:

	Pros	Cons
OB	OB has different pharmacological properties that acting in synergy can contribute to its spasmolytic effect: - L-Type VDCC inhibition - T-Type VDCC inhibiton - Antimuscarinic -Antitachykinergic	OB is not absorbed and therefore is devoid of systemic effects (Evangelista *et al.*, 2000): the effect is limited to the colonic wall.
H_2S	H_2S has different pharmacological properties that acting in synergy can contribute to its spasmolytic effect: -Hyperpolarization and relaxation of smooth muscle - Antimuscarinic - Antitachykinergic	Lack of a well-defined receptor or mechanism of action. H_2S may target too many receptors and pathways ("promiscuity")(Jimenez, 2010). H_2S can stimulate secretion and consequently increase colonic motility (Schicho *et al.*, 2006;Hennig & Diener, 2009).
$P2Y_1$ agonists	α,β-meATP mimics the endogenous purine and can be a good complement of NO in order to treat hypercontractility.	α,β-meATP may possibly activate other P2 receptors such as P2X that may be present in afferent neurons, glia, SMCs, ICCs or PDGFRα+ cells. P2X receptors can activate nociceptive mechanisms (Xue *et al.*, 1999;De Man *et al.*, 2003;Burnstock, 2014a;Burnstock, 2012;Peri *et al.*, 2013). The endogenous $P2Y_1$–mediated response can suffer a rundown (Mane *et al.*, 2014).
PGE_2	EP_2 and EP_4 receptor activation leads to smooth muscle relaxation and might be a pharmacological target to treat hypermotility. EP_2 and EP_4 antagonists could be used to treat hypomotility associated to over-expression of PGE_2 during gut inflammation.	PGE_2 can also activate EP_1 and EP_3 contractile receptors (Narumiya & FitzGerald, 2001;Sugimoto & Narumiya, 2007). PGE_2 can activate EP receptors located in the mucosa and lead to secretion (Dey *et al.*, 2006).

PGs, including PGE_2, may accomplish a relevant function not only in the regulation of physiological processes in the GI tract such as motility but also in several diseases such as IBD or colorectal cancer (Wang & DuBois, 2008;Dey *et al.*, 2006). Several studies have given evidence of the link between prostanoids and inflammatory gut processes; for instance, IBS patients present higher arachidonic acid plasma levels when compared to controls (Clarke *et al.*, 2010). Moreover, an up-regulated EP receptor expression has been associated to colonic inflammation and neoplasm (Takafuji *et al.*, 2000;Narumiya & FitzGerald, 2001;Dey *et al.*, 2006;Wang & DuBois, 2008;Sugimoto & Narumiya, 2007). Therefore, if PGE_2 (and probably other derivatives from arachidonic acid) and also EP receptors are over-expressed in inflammatory disorders, it is reasonable to believe that prostanoid mediated pathways can contribute to the associated impairment of gut contractility. Indeed, the blockade of EP_2 and

EP_4 receptors as well as inhibition of COX-2 has demonstrated to considerably improve the motility dysfunction associated to colonic obstruction (Lin *et al.*, 2012). The findings reported in the present work have demonstrated that both EP_2 and EP_4 receptors mediate the inhibitory effects of PGE_2 in mouse colonic motility in healthy state. Moreover, we have also reported a depolarization and a considerable increase on spontaneous contractility when both receptors are blocked. These findings suggest a constitutive production of PGs in the mouse colon that, when acting on EP_2 and EP_4 receptors, contribute to the maintenance of an inhibitory smooth muscle tone in physiological state and that their possible over-expression during gut inflammation may contribute to dismotility. In our study, the EP_2 and EP_4 receptor antagonists used (PF-04418948 and L-161,982 respectively) have demonstrated potent and selective effects. A prior in vivo study has also demonstrated that orally administered PF-04418948 can effectively block cutaneous blood flow mediated by EP_2 receptors in rats (af Forselles *et al.*, 2011). These findings should be considered a preliminary step to keep on investigating the potential therapeutic benefits of EP receptor antagonists as well as the implication of prostanoid related pathways in gut motility disorders.

$P2Y_1$ receptor KO mice present an important impairment of GI motility, giving evidence of the relevance of purinergic neurotransmission in gut function (Hwang *et al.*, 2012). Several studies have studied the relevance of a defective purinergic neurotransmission in animal models of GI inflammation (Strong *et al.*, 2010;Roberts *et al.*, 2012). We believe that $P2Y_1$ receptors have crucial physiological relevance and that $P2Y_1/sK_{Ca}$ pathways are potential endogenous mechanisms to modulate motility. Purine receptors might be a potential pharmacological target in gut motility disorders. Since purines together with NO are the main inhibitory NTs in the colon (Gallego *et al.*, 2008b;Grasa *et al.*, 2009;Gallego *et al.*, 2011;Jimenez *et al.*, 2014), $P2Y_1$ agonists might be a useful complementary treatment to NO donors, which have already proved their utility in the treatment of hypertonic sphincter in anal fissure (Collins & Lund, 2007). However, some of the purinergic agonists tested in vitro in the colon reduce contractility only in a phasic and transient manner, as it may be the case of the $P2Y_1$ agonist MRS2365 (Gallego *et al.*, 2011). The present work has reported that both in the rat and the human colon αβ-meATP is the purine that better mimics the endogenous purine despite the multiple receptors or other possible targets that could be activated when a drug is exogenously added. Moreover, the duration of the inhibitory effect on mechanical activity elicited by the putative $P2Y_1$ agonist αβ-meATP was longer. Thus, although further investigation is needed to define the possible therapeutic potential of $P2Y_1$ related pathways in

the GI tract, αβ-meATP might be a putative future therapeutic tool in order to treat gut motility disorders.

Preliminary findings have revealed that H_2S may probably exert antinociceptive actions (Linden, 2014;Distrutti *et al.*, 2006) and beneficial effects in a model of rat colitis (Wallace *et al.*, 2009). Similarly to the hypomotility associated to the increased endogenous production of NO during inflammation (Auli & Fernandez, 2005;Porras *et al.*, 2006), a decreased production of H_2S seems to be related to hypermotility (Liu *et al.*, 2013). Thus, it should be investigated if an increased production of H_2S happens during inflammation, and if that feature contributes to hypomotility in a similar way to NO.

The present work has also reported a blockade of cholinergic and tachykinergic contractions by the H_2S donor NaHS. It is widely known that ACh is the main excitatory NT in the gut and that TKs may also have an important role in gut motility (Costa *et al.*, 2000;Holzer & Holzer-Petsche, 2001;Lecci *et al.*, 2002). Indeed, anticholinergic drugs are commonly used as antispasmodics in IBS treatment (Drossman *et al.*, 2002), and both NK_2 antagonists and antimuscarinics significantly reduce propulsive contractions in the guinea-pig colon (Tonini *et al.*, 2001). Thus, it may be reasonable to postulate that H_2S may be a potential antispasmodic agent too, and that H_2S donors might be useful pharmacological tools in a similar way to NO donors. Indeed, NaHS shares some pharmacological properties with the other compound studied in the present work: OB. However, OB blocks EFS or agonist induced cholinergic and tachykinergic contractile responses with a higher potency when compared to the H_2S donor NaHS. Moreover OB is considered a safe and efficacious drug (Forte *et al.*, 2012). So, despite the potentially spasmolytic effect of H_2S, it is important to take into account the possible side effects that H_2S may have. In this way, it has been reported that H_2S donors evoke an important stimulation of secretion (Schicho *et al.*, 2006;Hennig & Diener, 2009), which may lead to an increase of colonic luminal volume and the consequent stimulation of contractility. Moreover, important systemic effects as significant reduction in blood pressure (Tang *et al.*, 2013) could appear if the colonic epithelial barrier was damaged due to concomitant inflammation. Further investigation is needed in order to characterize the safety and the possible therapeutic uses of H_2S donors in the GI tract.

OB is an efficacious and safe spasmolytic drug currently used in the treatment of dismotility related to GI inflammation. Although further investigation is needed, EP_2/EP_4 receptor, $P2Y_1$ receptor and H_2S related pathways might also be considered as potential future pharmacological targets to treat gut motility disorders.

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CONCLUSIONS

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1. $P2Y_1$ receptors and sKca mediate the purinergic endogenous response responsible for smooth muscle hyperpolarization and relaxation both in human and rat colon. Based on this criteria, we have demonstrated that the synthetic ATP analog α,β-meATP is the purine that better mimics the endogenous purinergic transmitter in both species. According to our results, although αβ-meATP has been classically considered a P2X agonist, it may also be able to directly or indirectly activate $P2Y_1$ receptors.

2. The identity of the purinergic transmitter is still under debate. It is possible that a wide variety of purines including ATP and β-NAD as well as their metabolites (ADP and ADP-ribose respectively) participate in purinergic inhibitory neuromuscular responses. It might be possible that the purinergic neurotransmission is not mediated by a single molecule and that multiple purines may be targeting multiple cell targets in the neuroeffector syncytium in smooth muscle. Further research is needed in order to determine the nature of the purine(s) involved in the gastrointestinal relaxation and how all these substances engage in co-regulation of effector systems.

3. Both EP_2 and EP_4 receptors independently mediate PGE_2 induced hyperpolarization and relaxation of mouse circular colonic smooth muscle. Our findings also suggest that constitutively produced prostaglandins, probably PGE_2, contribute to the maintenance of a smooth muscle inhibitory tone by activation of both EP_2 and EP_4 receptors.

4. In “non-nitrergic, non-purinergic” conditions, short trains (1 s) of EFS induce an atropine sensitive cholinergic contraction whereas long trains (10 s) of EFS induce an atropine resistant tachykinergic contraction in the human colon. We propose that these experimental conditions may be suitable to test drugs with potential spasmolytic actions associated to a reduction of excitatory neuronal inputs to smooth muscle.

5. According to previously reported findings and to the results presented in the present work, H_2S fulfils, at least partially, the criteria to be considered a gasomediator in the colon. It is possible that endogenously produced H_2S contributes to the maintenance of smooth muscle inhibitory tone in the human colon. H_2S may also exert an inhibitory control on cholinergic and tachykinergic neuromuscular transmission. Further investigation is needed in order to define the real physiological relevance of endogenous H_2S in the gastrointestinal tract.

6. In the present study we have demonstrated that the spasmolytic compound OB efficiently blocks L-type and T-type voltage dependent calcium channels, and muscarinic and tachykinergic receptors both in human colonic smooth muscle cells and in the rat colon. These four different mechanisms of action acting in synergy might explain the effectiveness of OB in the treatment of colonic motor disorders.

7. Our results suggest that the inhibitory pathways related to $P2Y_1$ receptors, EP_2 and EP_4 prostanoid receptors and H_2S should be considered as potential pharmacological targets that produce smooth muscle relaxation and hence could be useful tools to treat spasticity in colonic motor disorders. Further investigation is needed in order to find out their real therapeutic potential.